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(54) Title: HYBRID Fc RECEPTOR MOLECULES

(57) Abstract

A novel immunoglobulin binding molecule is provided being a hybrid of two or more binding molecules such as to provide a hybrid immunoglobulin binding molecule having chimeric properties characteristic of two or more individual immunoglobulin binding molecules in one molecule. Methods of production and applications of such hybrid molecules is also provided.

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TITLE: Hybrid Fc Receptor Molecules

This invention relates to immunoglobulin binding molecules and in particular to Fc receptor (FcR) molecules and hybrids or chimeric forms thereof capable of binding one or a plurality of classes of antibody.

The invention also encompasses the different forms of Fc receptor molecules including soluble, unbound and bound forms as protein molecules and nucleotide sequences coding therefore, methods of production of hybrid Fc receptor molecules, uses of hybrid Fc receptor molecules including diagnostic testing and pharmaceutical application, poly and monoclonal antibodies detecting hybrid Fc receptor molecules and the uses thereof.

To date the Fc receptor molecules and cDNA clones thereof have been limited to specific antibody class targets for example IgG, IgE, etc.

Receptors for immunoglobulin (Fc receptors or FcR) play key roles in the immune response in allergy and in resistance to infection (1-3). On phagocytes they are responsible for the binding and removal of immune complexes. On T and B cells they are involved in signalling and in the regulation of antibody synthesis (1,2). Secreted Fc receptor related molecules (immunoglobulin binding factors) have been defined that also regulate antibody synthesis and membrane bound or soluble FcR play a role in T cell function (2). The role of FcR in allergy has also been elucidated and the binding of IgE to its receptor plays a pivotal role in these conditions (3).

In addition, to this wide range of function, FcR receptors for all immunoglobulin classes have been defined. In man and the mouse two classes of receptors for IgG have been defined and are designated Fc γ RI, Fc γ RII. In addition a third class of receptor Fc γ RIII has been identified in man. These receptors can be distinguished by (i) structural differences, (ii) affinity of binding of antibody, (iii) specificity for Ig classes or subclasses and (iv) reaction with monoclonal antibodies to FcR. Thus, Fc γ RI is a high affinity receptor for monomeric IgG whereas Fc γ RII and Fc γ RIII bind monomeric IgG with low affinity but bind immune complexes with high avidity (1-2).

Molecular cloning of cDNA or genes encoding these receptors has demonstrated that they are homologous proteins that have evolved to perform their

unique functions (4,5,6,7). Indeed these cloning experiments have shown that not only are there multiple forms of each of Fc γ RI, Fc γ RII in man and mouse (and Fc γ RIII in man) but that Fc γ Rs are related (4-10). Furthermore, the receptors for IgG and IgE are also related at the nucleotide and protein levels (5,6,11,12).

The interaction of immunoglobulins with FcR was investigated by constructing chimeric Fc γ R. Mouse Fc γ RI and Fc γ RII cDNAs have been cloned and encode related but distinct proteins. Fc γ RI and II are both typical membrane spanning proteins (4,5,7). The extracellular region of Fc γ RI is organised into three disulphide bonded domains. By contrast, the extracellular region of Fc γ RII is composed of only two disulphide bonded domains. Domains 1 and 2 (D1 and D2) of Fc γ RI are homologous to domains 1 and 2 (d1 and d2) of Fc γ RII showing approximately 47% amino acid homology and D3 of Fc γ RI is unique.

In addition to the differences in structure between Fc γ RI and Fc γ RII these receptors differ in their specificity and affinity for IgG. Fc RI binds only mouse IgG2a with high affinity whereas Fc γ RII binds mouse IgG1, IgG2a and IgG2b.

Thus in vitro mutagenesis was used to determine functional regions of these Fc γ R and to generate chimeric Fc γ R with the combined properties of these receptors.

In addition in vitro mutagenesis was performed to generate hybrid FcR composed of functional regions of Fc γ RII and Fc ϵ RI wherein such a hybrid R c R possess the properties of both Fc γ RII and Fc ϵ RI.

Accordingly the invention provides a hybrid FcR molecule capable of binding to any one or more classes of antibody molecules.

Preferably the hybrid FcR comprises one or more functional domains of FcR linked to the structural or functional region of another molecule.

Preferably the hybrid FcR molecule binds to one of antibody classes IgM, IgG, IgA, IgD or IgE.

Preferably the hybrid FcR molecule is in soluble form.

Preferably the hybrid FcR molecule contains domains D1 and D2 of Fc γ RI linked to the transmembrane region and cytoplasmic tail of Fc γ RII and is designated Fc γ RI/II.

Preferably the hybrid FcR molecule contains domain d1 and d2 of Fc γ RII

linked to domain D3, transmembrane region and the cytoplasmic tail of Fc RI and is designated Fc γ RII/I.

Preferably the hybrid Fc γ R is human FcR.

Preferably the hybrid FC γ R is mouse FCR.

Preferably the IgE binding region of IgE FcR is linked or located within Fc γ RI.

Preferably the IgE binding region of IgE Fc ϵ R is linked or located within Fc γ RII.

The invention further provides a hybrid FcR wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from Fc γ RII having the following amino acid sequence :-

- Phe Ser Arg Leu Asp Pro Thr Phe Ser Tle Pro Gln Ala Asn His Ser His Ser Gly Asp Tyr His Cys Thr Gly Asn Ile Gly Tyr Thr Leu Phe Ser Ser Lys Pro Val Thr Ile Thr Val, or functional equivalent thereof.

The invention further provides a hybrid FcR wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from Fc ϵ RI having the following amino acid sequence :-

- Trp Leu Leu Leu Gln Ala Ser Ala Glu Val Val Met Glu Gly Gln Pro Leu Phe Leu Arg Cys His Gly Trp Arg Asn Trp Asp Val Tyr Lys Val Ile Tyr Tyr Lys Asp Gly Glu Ala Leu Lys, or functional equivalent thereof.

Preferably the FcR is soluble or membrane bound.

The invention further provides:

A hybrid FcR being a chimera comprising a first extracellular domain of Fc γ RII, a second extracellular domain of Fc ϵ RI and a transmembrane region and cytoplasmic tail of Fc γ RII;

A hybrid FcR being a chimera comprising a first extracellular domains of Fc ϵ RI containing amino acids 1 - 169 of Fc ϵ RI linked to amino acids 170-281 of Fc γ RII;

A hybrid FcR being a chimera comprising a first extracellular domain of Fc ϵ RI being amino acids 1-86 linked to a second extracellular of Fc γ RII being amino acids 87-169 linked to a transmembrane region and cytoplasmic tail of Fc γ RII being

amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of Fc ϵ RI containing amino acids 1-86 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 87-128 linked to part of the second extracellular domain of Fc γ RII being amino acids 129-169 linked to a transmembrane region and cytoplasmic tail of Fc γ RII being amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of Fc ϵ RI being amino acids 1-86 linked to part of the second extracellular domain of Fc γ RII being amino acids 87-128 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 129-169 linked to a transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of Fc γ RII being amino acids 1-86 linked to part of the second extracellular domain of Fc γ RII being amino acids 87-128 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281;

A hybrid FcR being a chimera comprising the first extracellular domain of Fc γ RII being amino acids 1-86 linked to the second extracellular domain of Fc ϵ RI being amino acids 87-169 linked to a transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281;

A hybrid FcR being a chimaera comprising the first extracellular domain of Fc γ RII being amino acids 1-86 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 87-128 linked to part of the second extracellular domain of Fc γ RII being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281.

The invention further provides amino acid and nucleotide sequences for hybrid FcR, polyclonal and monoclonal antibodies raised thereto, biological products incorporating FcR hybrids, diagnostic kits and assays and pharmaceuticals.

The invention further provides a method of generating hybrid FcR molecules comprising linking different functional domains of different Fc receptor molecules to construct hybrid FcR molecules.

The invention further provides a method of generating hybrid FcR molecules comprising ligating a first FcR molecule at the transmembrane junction, attaching a second FcR molecule to excise suitable domain regions and linking these two fragments to construct a hybrid Fc receptor molecule.

To generate these receptors an Apa-I restriction site was introduced into the cDNA sequence of Fc γ RI at the junction of sequences encoding D2 and D3. Fc γ RII cDNA already has an Apa site at the junction of d2 and the membrane spanning region. Thus the introduction of this restriction site (or any convenient restriction site) would facilitate the exchange of sequences between these or other receptors.

To introduce the Apa I site into the Fc γ RI cDNA two separate PCR mutagenesis reactions were performed and each used oligonucleotide primers containing an Apa I recognition sequence (Fig. 1) (13-15). The template for PCR was the Fc γ RI cDNA cloned into the pGEXII expression vector (7). PCR amplification of sequences Fc γ RI D1 and D2 was primed using a oligonucleotide containing Sal I site that hybridized to the leader sequence at the 5' end of the cDNA and the second primer MDH1 which hybridized between D2 and D3 and would introduce an Apa I site between nucleotides 591 and 592 by altering three nucleotides of the Fc γ RI sequence GAGCTC to GGGCCC (Fig. 1). The second PCR amplified the sequence containing D3, the transmembrane domain and cytoplasmic tail. A primer (MDH2) also containing an Apa I recognition sequence hybridizes to the sequences between D2 and D3 of Fc γ RI in combination with the second oligonucleotide (MDH4) which contained a Sal I site. This primer hybridizes to the pGEXII vector sequences at the 3' end of the Fc γ RI cDNA insert.

The PCR products were then digested with Apa I or Sal I and ligated into Fc γ RII expression systems as outlined below.

To construct expressable FcR chimeric cDNA, Fc RII cDNA was subcloned into the PstI site of the expression vectors pKC3 or pKC4 downstream of the SV40 early promoter. These vectors differ only in the orientation of the polylinker and provided a unique Sal I site at the 5' or 3' end of the Fc γ RII cDNA (Fig. 2a). The pKC3-FcRII and pKC4-FcRII cDNA were digested with Sal I and Apa I to remove Fc γ RII d1 and d2 or the transmembrane and cytoplasmic encoding regions from these

vectors (Fig. 2b, 2c). The PCR products containing Sal I and Apa I sticky ends and encoding D1 and D2 of Fc γ RI (generated above; (Fig. 1)) were then subcloned into Sal I/Apa I digested pKC3-Fc γ RII. Similarly, to generate a hybrid cDNA encoding d1 and d2 of Fc γ RII linked to D3, and transmembrane and cytoplasmic tail of Fc γ RI. The PCR products containing D3, the transmembrane and cytoplasmic regions of Fc γ RI were linked to the D1 and D2 of Fc γ RII in the pKC4-Fc RII (Fig. 2e).

The chimeric cDNA were checked for the correct sequence by nucleotide sequencing. As expected the Fc γ RI/II chimera contained Fc γ RI nucleotide sequence from positions 9 to 594 that encodes Fc γ RI D1 and D2 and Fc γ RII cDNA sequence from positions 595 to 1244 that encode the transmembrane and cytoplasmic region of Fc γ RII (Fig. 3a). The Fc γ RII/I chimera contained Fc γ RII nucleotide sequence from positions 1-662 (encoding d1 and d2) and Fc γ RI sequence from positions 663-1348 that encode D3, the transmembrane and cytoplasmic tail of Fc γ RI (Fig. 3b).

The specificity of the chimeric Fc γ R and Fc γ RI and Fc γ RII were then tested in a transient expression system by transfecting the cDNA clones into monkey COS cells. The binding of rabbit IgG and mouse Ig subclasses was then assessed by EA rosetting. As expected rabbit IgG bound to cells transfected with Fc γ RI (Figure 4a) and Fc γ RII (Figure 4b) and also bound to both chimeric Fc γ R i.e. Fc γ RI/II (Figure 4c) and Fc γ RII/I (Fig. 4d, Table 1). Thus the D1 and D2 of Fc γ RI (now anchored in the cell membrane by the membrane spanning region of Fc γ RII) have the capacity to bind IgG in the absence of D3 of Fc γ RI.

Similarly, d1 and d2 of Fc γ RII retain the Ig binding capacity when associated with domain 3 of Fc γ RI (Fig. 1b, Table 1). Since Fc γ RI and Fc γ RII differ in their specificity for mouse IgG subclasses (Fc γ RI binds IgG2a but Fc γ RII binds IgG1, IgG2a, IgG2b (ref 1-10)) it was of interest to determine the specificity of the chimeric Fc γ R molecules for mouse IgG subclasses. As expected cells transfected with the native Fc γ RI bound IgG2a sensitised erythrocytes (Figure 4f) but did not bind IgG1 (Figure 4g) and cells transfected with vector only did not bind IgG-EA of any class (Figure 4h, Table 1). The Fc γ RI/II chimaera (with the extracellular region consisting only of D1 and D2 of Fc γ RI) not only bound IgG2a (Figure 4i) but also

bound IgG1 (Figure 4j) and IgG2b (Table 1), which do not bind to native Fc γ RI (Table 1; refs 1-10) i.e. these Fc γ RI domains have now lost their unique specificity for IgG2a and IgG2b and have acquired the specificity of Fc γ RII. Thus, the specific binding of IgG2a by normal Fc γ RI involved D1 and D2 as well as D3 since the removal of D3 (by generation of the chimeric Fc γ RI/II) results in a broadening of the specificity of these domains. Thus by engineering the cDNA we were able to generate receptors of altered specificity i.e. convert Fc γ RI to Fc γ RII-like function.

Examination of the chimeric Fc γ R (Fc γ RII/I) containing d1 and d2 of Fc γ RII linked to D3, the transmembrane and cytoplasmic region of Fc γ RI, showed that this molecule bound IgG1, IgG2a and IgG2b, i.e. the specificity of d1 and d2 of Fc γ RII was retained. Furthermore, the presence of D3 of Fc γ RI did not influence the binding of the various mouse IgG subclasses to this chimeric Fc γ R.

The chimeric FcR proteins expressed on the surface of transfected cells were tested for reactivity with the 2.4G2 monoclonal antibody which reacts with Fc γ RII but not with Fc γ RI.

It is known that this antibody completely inhibits Ig binding to Fc γ RII and does not bind to Fc γ RI.

Transfected cells expressing normal and chimeric Fc γ R were pretreated with Fab fragments of the monoclonal antibody 2.4G2 and then tested for their ability to bind rabbit IgG coated erythrocytes. The Fab fragments completely inhibited binding of antibody coated erythrocytes to Fc γ RII and the chimeric Fc γ RII/I (Table 2). In addition, the 2.4G2 Fab₂ did not inhibit binding to Fc γ RI but by contrast completely inhibited binding to the chimeric Fc γ RI/II containing D1 and D2 Fc γ RI (Table 2). Thus D1 and D2 of Fc γ RI now express the 2.4G2 when not associated with D3 of Fc γ RI.

To further define the role of the Fc γ RI extracellular domains, the binding of monomeric IgG to the chimaeric receptors was investigated. The affinity of monomeric IgG binding was assessed by Scatchard analysis with ¹²⁵I labelled monoclonal antibodies of IgG2a subtype on transfected COS cells expressing either of the two chimaeric receptors, native Fc γ RI or Fc γ RII. The high affinity receptor Fc γ RI bound IgG2a with affinity $1.4 \times 10^8 M^{-1}$ (Figure 5) which is similar to that

reported previously (1-3,7). In contrast, the binding of monomeric IgG2a by the Fc γ RI/II and Fc γ RII/I chimaeric receptors, and the low affinity receptor Fc γ RII (as expected), was not detectable (not shown).

Furthermore, high affinity binding was not able to be generated by linking D3 of Fc γ RI to d1 and d2 of Fc γ RII, despite the high homology of Fc γ RII d1-d2 with Fc γ RI D1-D2. This suggests that all three extracellular domains of Fc γ RI are required for the high affinity interaction with IgG2a, D3 being crucial in modifying the D1-D2 interaction with IgG binding properties of Fc γ RII, as they do not detectably bind monomeric IgG2a and also show the broader specificity for IgG1, 2a, 2b immune complexes that is characteristic of Fc γ RII. Clearly these domains represent a conserved Fc γ RII-like IgG binding unit between these otherwise functionally distinct receptors. However, there must be specialised differences between the homologous two domain structure of Fc γ RI and Fc γ RII, as only D1 and D2 of Fc γ RI, not d1 and d2 of Fc γ RII, have the capacity to interact with D3 to produce the specific high affinity binding of IgG2a.

It should be noted that the strategy used to generate the Fc γ RII/I chimaeric cDNA led to the replacement of the glutamic acid and leucine residues at positions 172 and 173 with glycine and proline, through the introduction of an Apal site at the junction of D2 and D3 of Fc γ RI. To ensure that these amino acid substitutions were not responsible for the observed properties of the Fc γ RII/I chimaeric receptor, mutagenesis was used to replace the glycine and proline residues with the original glutamic acid and leucine residues. This chimaeric receptor exhibited identical IgG binding characteristics to the original Fc γ RII/I chimaera (data not shown).

Based on findings presented herein, it is possible to make several statements on the origin of Fc γ RI which highlight important aspects regarding evolution of Fc γ R genes and the Ig superfamily. Given that there is considerable amino acid homology between D1-D2 of Fc γ RI and d1-d2 of Fc γ RII(7), and that D1-D2 adopts the specificity and affinity for IgG of Fc γ RII after the removal of D3, it is clear that the two receptors have a common evolutionary history. Indeed, the recent cloning and mapping of multiple mouse and human Fc γ RII genes, multiple human Fc γ RI genes and Fc γ RIII genes, and both the mouse and human Fc ϵ RI genes, indicate there has

been considerable gene duplication in the same region of chromosome 1 to produce this subfamily of Ig related molecules which show great amino acid homology (16-19).

The substantial amino acid identity between the extracellular regions of IgG receptors, (approximately 90% within a class e.g. Fc γ RIIa as compared to Fc γ RIIb1, Fc γ RIIb2 and Fc γ RIIb3 or Fc γ RIII-1 compared to Fc γ RIII-2) or between the extracellular regions between FcR classes (e.g. approximately 50% between Fc γ RI and Fc γ RIII and 40% between Fc γ RII and Fc ϵ RI) indicates shared structure although there are clear differences in function or specificity (4-12). This high conservation of amino acid identity between FcR is also seen between the low affinity IgG Fc γ RII and the high affinity IgE receptor Fc ϵ RI. Although these receptors have very distinct specificity for immunoglobulins, Fc γ RII binds IgG whereas Fc ϵ RI binds IgE, these FcR share approximately 40% amino acid identity in their Ig binding, extracellular domains.

Thus it should be possible to generate many novel chimaeric Fc receptors that contain parts and therefore characteristics and functions of multiple receptor classes e.g. a chimaeric FcR composed of Fc γ RII and Fc ϵ RI sequences that binds IgG and IgE; a chimaeric Fc γ R composed of parts of Fc γ RII and Fc γ RI or Fc γ RII and Fc γ RIII, or Fc α R (IgA receptors) and Fc γ RII or other FcR. Similarly these chimeric receptors may be composed of sequences derived from three or more Fc receptors e.g. Fc γ RII and Fc γ RI and Fc ϵ RI or Fc γ RI and Fc α R and Fc ϵ RI and Fc μ R (IgM receptors) etc.

The origin of the third domain in the extracellular region of Fc γ RI is also of interest, unlike Fc γ RII and Fc γ RIII, Fc γ RI is unique in that it has an additional extracellular domain. Whether D3 of Fc γ RI arose by duplication of a related exon or by an insertion of an exogenous exon is not clear, as sequence comparisons indicate D3 is only distantly related to D1 and D2, or to domains of other Ig superfamily members. However the data presented herein demonstrates how Ig-superfamily genes may have evolved, in that Fc γ RI probably originated through the acquisition of an exon encoding an intact Ig like domain (D3), presumably by a primordial Fc γ RII-like gene, with subsequent divergence refining the interaction of the three

domains to produce a new receptor with modified IgG binding characteristics i.e. a specialised high affinity receptor for IgG2a. This model for Fc γ RI evolution is consistent with the proposed evolution of the Ig superfamily postulated by Williams and colleagues, wherein primordial genes acquire individual exons encoding Ig-like domains, and these domains become functionally incorporated to create new molecules with new function. Indeed, each of the extracellular Ig-like domains of mouse Fc γ RI and Fc γ RII are encoded by individual exons (N. Osman, M. Hogarth unpublished observations, Ref. 19).

Finally, based on the high homology between D1, D2 and D3 of human and mouse Fc γ RI (69,74 and 75% amino acid identity respectively (7), together with the observation that human Fc γ RI also binds mouse IgG2a with high affinity (10), it is likely that the contributions of the extracellular domains of human Fc γ RI to IgG binding will parallel those reported here for mouse Fc γ RI and Fc γ RII.

Clearly it is possible to generate functional chimeric FcR and several statements can be made. First chimeric cDNA clones encoding FcR composed of components of different Fc γ R have been generated. Second, by connecting D1 and D2 of Fc γ RI to the transmembrane cytoplasmic regions of Fc γ RII a receptor molecule was produced that has a broader specificity than the receptor from which the Ig binding regions were derived, i.e. Fc γ RI/II containing D1 and D2 of Fc γ RI binds mouse IgG1, IgG2a, IgG2b. Thirdly, similar experiments that generate chimeric Fc γ R between human Fc γ RII and human Fc γ RI have shown that these chimeric receptors have altered specificity for IgG. Fourthly, it is possible to generate numerous other chimeric FcR that possess characteristics of other FcR. Since most FcR are homologous proteins it is possible to insert/or attach Ig binding regions to Fc γ RI or II e.g. insert the IgE binding region of IgE FcR (Fc ϵ RI) into another FcR e.g. Fc γ RII.

Indeed, the following experiments describe the production of chimaeric receptors containing amino acid sequence elements of the human low affinity Fc γ RII and the human high affinity IgE receptor Fc ϵ RI. These chimeras were generated by exchanging parts of the cDNA sequence encoding the regions shown in the Figures 6,7 and Tables 3,4. Although the IgG and IgE receptors have distinct biological

properties and specificities they are homologous transmembrane glycoproteins, both containing two extracellular immunoglobulin binding domains (EC domains) which have approximately 40% amino acid identity. This considerable identity presumably indicates that the extracellular portions of these distinct molecules would be expected to have a similar general structure. Chimeric receptors formed between these classes of receptors would therefore be expected to be expressed on the cell surface, processed appropriately and indeed the transfection experiments indicate that this is the case (Figure 8, Table 5). We have successfully isolated and transfected the human Fc γ RII cDNA defined in Australian Patent Serial No. 595623 (reference 6) and the cDNA sequence is shown in Figure 6. The human high affinity receptor for IgE has been previously described (12) and the sequence is shown in Figure 7. This receptor is expressed extensively on mast cells and basophils and shows a high affinity for IgE. Biochemical studies have indicated that the Ig binding subunit (α subunit) (sequence shown in Figure 7) is associated with two additional subunits, the β subunit and a dimer of two γ subunits that are disulphide bonded to one another. Expression of the α subunit normally requires the expression of one or more of the β or γ subunits (21). The strategy employed to examine the Ig binding characteristics of the extracellular domains of Fc γ RII and Fc ϵ RI was to exchange the entire domains or parts of domains between two types of receptors and examine the specificity of these receptors with respect to the binding of IgG and IgE. The nucleotide sequence of all chimeric cDNA described herein (Table 3,4) was confirmed by nucleotide sequencing.

Since the Fc ϵ RI subunit requires the presence of additional subunits for expression and the subunits are not synthesised in the COS cells (which are used as the target cells for transfection and expression experiments) an Fc ϵ RI α chain capable of being expressed alone was generated. The initial strategy involved placing the entire extracellular domains of Fc ϵ RI on the membrane spanning and cytoplasmic region of Fc γ RII. This was performed by splice overlap extension (SOE) using oligonucleotides EG6 and EG1 (Table 4) which hybridise to the Fc ϵ RI cDNA (Figure 7) to generate amplified Fc ϵ RI sequence and the oligonucleotide pair EG2 and EG5 (Table 4) which hybridise to the Fc γ RII cDNA (Figure 6) to generate the amplified

Fc γ RII sequence. Since sequence within EG1 and EG2 overlap, the two amplified products were used in the overlap extension to produce the full length recombinant Chimaera 1 (Table 3). Thus a chimaeric cDNA was generated which encoded the 25 amino acids of the Fc ϵ RI leader sequence and 169 amino acids of the extracellular portion of Fc ϵ RI which was linked to the amino acid sequences from position 170 in Fc γ RII to the C terminal amino acid as position 281 of Fc γ RII (Table 3 and 4).

To establish if this chimeric cDNA encoded a functional IgE receptor the chimeric cDNA contained in expression vector pKC3 (Van Doren et al., J. Virol. 50:606, 1984) was transfected into COS cells in a transient expression system and expression measured 48-72 hours later. Expression was tested by EA rosetting using IgE sensitised erythrocytes and by a direct binding assay using Scatchard analysis using 125 iodine labelled human IgE. Cells transfected with the Chimaera 1 cDNA bound IgE immune complexes, [IgE sensitized erythrocytes (Figures 8a)] and also bound monomeric human IgE with high affinity, but did not bind IgG sensitised erythrocytes (Figures 8d, Table 5) as would be expected of an Fc ϵ RI. Conversely, Fc γ RII bound IgG-EA (Figure 8b) but not IgGE-EA (Figure 8A) as expected. This experiment establishes that the expression of Chimaera 1 and the interaction with and specificity for IgE of the Ec domains of Fc ϵ RI are not dependent on the additional subunits or the membrane spanning region of Fc ϵ RI.

In the light of experiments using FcR, with the high and low affinity IgG receptors (described earlier) a series of chimeric cDNAs and Fc receptor proteins containing Fc γ RII and Fc ϵ RI sequences were then produced in order to:-

1. Establish the extent to which replacement of extracellular sequences from one receptor with those of a second receptor would enable the production of functional Fc receptors.
2. Generate functional receptors that have properties of multiple Fc receptors e.g. Fc receptors that bind both IgG and IgE.
3. Localise the regions of Fc receptors responsible for the interaction with immunoglobulins.

To determine the functional Fc binding domain(s) of Fc ϵ RI and Fc γ RII, chimaeric receptors were generated such that they contained two extracellular

domains (EC domains), one of which was derived from Fc ϵ RI, the second of which was derived from Fc RII (Table 3,4,5). Chimaera 2 contained the first extracellular domain of Fc γ RII and the second domain of Fc ϵ RI organised in that order. This chimaera was generated by splice overlap extension (SOE) using the oligonucleotides NR1 and EG11 as well as EG10 and EG5 to respectively amplify the appropriate sequences from Fc γ RII cDNA or from the Chimaera 1 cDNA (Table 4, Figures 6,7). The overlap extension reactions were performed as detailed in the Materials and Methods and were possible because of the overlapping sequence contained in oligonucleotides EG10 and EG11. The resulting chimaeric cDNA (Chimaera 2) encoded a chimaeric Fc receptor containing the Fc γ RII leader sequence (amino acid-34 to the N-terminus) and the first EC domain of Fc γ RII amino acid 1 to 86 inclusive, constituting the first EC domain of the Chimaera 2. In addition the overlap extension reactions also included the second EC domain of FC ϵ RI corresponding to amino acids 87 to 169 of this receptor. As Chimaera 1 was used as a template for this region, Chimaera 2 also contains the membrane spanning region and cytoplasmic tail of Fc γ RII, which included amino acids 170 to 281 (Table 3). Similarly, the chimaeric Fc receptor (Chimaera 3) was generated to contain the first EC domain of Fc ϵ RI and the second EC domain, transmembrane region and cytoplasmic tail of Fc γ RII. Splice overlap extension was used to generate the chimeric cDNA that encoded this receptor by using oligonucleotide pair EG6 and EG9 on the Fc ϵ RI cDNA template and oligonucleotides EG8 and EG5 with Fc γ RII cDNA as a template. The splice overlap extension reactions were possible because of the overlapping sequence contained in oligonucleotides EG9 and EG8. The Fc receptor encoded by this chiaeric cDNA contained the leader sequence (amino acids-25 to -1 of Fc ϵ RI) as well as the first EC domain of Fc ϵ RI (amino acids 1 to 86) as well as the amino acids of the second EC domain, membrane spanning and cytoplasmic tail of Fc γ RII (amino acids 87 to 281 inclusive) (Tables 3,4 Figures 6,7).

To assess the specificity of these chimaeric receptors, the chimaeric cDNAs, cloned into the pKC3 expression vector, were transiently expressed by transfection into COS 7 cells. The capacity of these Fc receptors expressed on the transfected cells to bind IgG or IgE was tested by the binding of immune complexes in the form

of erythrocytes sensitised with IgG or IgE (IgG-EA or IgGE-EA). Chimaera 2 containing the first EC domain of Fc γ RII and the second EC domain of FC ϵ RI bound IgE but did not bind IgG sensitised erythrocytes (Figure 8e,f; Table 5). Conversely, Chimaera 3 containing the first EC domain of FC ϵ RI and the second EC domain of Fc γ RII bound IgG but not IgE immune complexes (Figure 8g,h). These results indicate that the second EC domain is intimately involved in the interaction with immunoglobulins since in Chimaera 2 the first EC domain was derived from the IgG receptor and the second from the IgE receptor but only bound IgE not IgG. Conversely, in Chimaera 3 the first EC domain was derived from the IgE receptor and the second from the IgG receptor and could clearly bind IgG but not IgE. Since the normal Fc RII and normal FC ϵ RI receptors exclusively bind IgG and IgE respectively (as does Chimaera 1) it is clear that the interaction of Chimaeras 2 and 3 with IgE and IgG respectively is mediated within the second EC domain of Fc receptors (Table 3).

To localise the binding of immunoglobulins to a subregions of EC domain 2 in both Fc γ RII and FC ϵ RII additional chimaeric receptors were generated wherein EC domain 1 was derived from either FC ϵ RI and Fc γ RII (Table 4). These chimeric receptors were generated using the splice overlap extension technique, where amplification of the appropriate regions of cDNA were performed on the wild type FC ϵ RI or Fc γ RII cDNA as well as the appropriate chimaeric cDNA (Table 3, 4). Chimaera 4 contained the first EC domain of FC ϵ RI together with a substantial amino terminal portion of the second EC domain of FC ϵ RI (amino acid -25 to -1 together with amino acids 1 to 128 inclusive). These amino acids were connected to the C-terminal portion of the second EC domain of Fc γ RII, and membrane/ cytoplasmic regions of Fc γ RII amino acids 129 to 281 inclusive (Table 3). This chimaeric cDNA was generated using the oligonucleotides EG6 with EG14 on the FC ϵ RI cDNA template, and EG15 together with EG5 on the Fc γ RII cDNA template (Table 4). COS cells were transfected with this chimaeric cDNA and the binding of IgE and IgG to the encoded Chimaera 4 receptor was examined by the binding of immune complexes in the form of antibody sensitized erythrocytes (Figure 8i, j; Table 5). These experiments indicated that the chimaeric Fc receptor Chimaera 4 bound both

IgG-EA and IgE-EA, this indicated that an IgG binding site could be localised to the C-terminal portion of the second EC domain within the region encoded by amino acids 129 to 169 inclusive. The observation that Chimaeria 4 bound IgE-Ea also indicates that an IgE binding region is located in the second domain in the region containing amino acids 87 to 128 inclusive. The involvement of these regions in the binding of IgE and IgG was verified by the construction of Chimera 5 and its subsequent transfection and immune complex binding studies.

Chimera 5 was generated by the splice overlap extension techniques using the oligonucleotides outlined in Table 4. This chimaera contained the first extracellular domain of Fc ϵ RI with the second EC domain composed of both Fc γ RII (amino acids 87 to 128 inclusive and Fc ϵ RI sequence (amino acids 129 to 169). Transfection of this cDNA indicated that the chimaeric Fc receptor failed to bind IgG or IgE (Tables 3 and 5). The amino acid sequence of Chimeras 4 and 5 differs only in the amino acid sequence within the second EC domain. Wherein amino acid residues, 87 to 128 inclusive, of Chimaera 4 have been derived from Fc ϵ RI and have been replaced with residues 87 to 128 of Fc γ RII in Chimaera 5. Since Chimaera 4 binds IgE and Chimaera 5 does not, residues (87-128) in Fc ϵ RI are important in IgE binding. Similarly as Chimaera 4 contains amino acid residues 129 to 169 inclusive, derived from Fc γ RII and binds IgG but Chimaera 5 which contains amino acids 129 to 169 inclusive, derived from Fc ϵ RI and does not bind IgG, indicates that these residues are important in IgG binding. Since Chimaeras 4 and 5 are half domain chimaeras ie. containing sequence from Fc ϵ RI and Fc γ RII within the second domain but have the same first EC domain derived from Fc ϵ RI, two additional chimaeras were made again containing the same amino acid configurations of the second EC domain as found in chimaeras 4 and 5, but containing the first EC domain derived from Fc γ RII. Chimaera 6 contains the first EC domain of Fc γ RII including the leader sequence corresponding to amino acids -34 to 1 and amino acids 1-86 inclusive as found in the first EC domain of Fc γ RII (Table 3, Figure 6). This was attached to a second EC domain wherein amino acids 87 to 128 inclusive were derived from Fc ϵ RI and amino acids 129 to 169 inclusive were derived from Fc γ RII (Figures 6,7). Transfection of this cDNA into COS cells showed that the encoded chimaeric

receptor could bind IgG and IgE (Table 3). By contrast, Chimaera 7 which contained the EC domain 1 of Fc γ RII but contained amino acids 87-128 of Fc γ RII and amino acids 129 to 169 of Fc ϵ RII but contained amino acids 87-128 of Fc γ RII and amino acids 129 to 169 of Fc ϵ RI failed to bind IgG or IgE when transfected into COS cells (Table 5). These results taken together with those experiments using the Chimeras 4 and 5 indicate that amino acids within the second domain are clearly important in the interaction of Fc receptors with Ig, and particularly amino acids 87-128 of Fc ϵ RI are important in the interaction with IgE and amino acids 129 to 169 of Fc γ RII in the interaction with IgG.

To further characterise the chimaeric receptor structures, monoclonal anti Fc γ RII antibodies (description of antibodies described below) were used in immunofluorescence studies on cells transfected with native Fc γ RII or chimaeric cDNAs. All monoclonal anti Fc γ RII antibodies bound to cells transfected with the native Fc γ RII cDNA (Table 5). As expected none of these antibodies bound to cells transfected with the native Fc ϵ RI α subunit or cells transfected with Chimaera 1, which contains the extracellular IgE binding domains (Table 5).

Analysis of monoclonal antibody (MAb) binding to the single domain chimaeras indicated that Chimaeras 2 and 3 have some obvious structural differences (Table 5). Monoclonal antibodies 8.7 and 7.30 detected the expression of Chimaera 3 but did not bind to Chimaera 2. Since Chimaera 3 contains EC domain 1 from Fc ϵ RI and EC domain 2 from Fc γ RII, this result indicates that the epitope on Fc γ RII molecules detected by 8.7 and 7.30 (see below) is located in Ec domain of Fc γ RII. By contrast, monoclonal antibodies 8.2 and 8.26 which bound to the native Fc γ RII failed to bind to any of the chimaeric receptors tested (Table 5), indicating that this epitope detected by these antibodies requires the presence of part or all the first and second EC domains and this clearly establishes the epitope detected by 8.2 and 8.26 as distinct from the epitope detected by antibodies 8.7 and 7.30. Of interest was the finding that the previously described (22) monoclonal antibody IV.3 bound to Chimaera 3, whereas a second previously described antibody CIKM5 (23) did not bind to any chimaeric cDNA. However, both antibodies bound to the native Fc γ RII.

The construction of these chimaeric receptors and the immune complex

binding studies as well as the characterisation using monoclonal antibodies has indicated several clear features of these receptors:-

- 1) The EC domains of Fc ϵ RI can be attached to the membrane spanning and cytoplasmic regions of a different molecule (Fc γ RII) and the binding of IgE to the extracellular domains of Fc ϵ RI is retained i.e. the extracellular domains function as receptors irrespective of additional sequence added at the C-terminal end of the EC domains. Clearly soluble forms of this receptor i.e. a form of the receptor containing only the extracellular domains or part thereof in the absence of a membrane spanning segment or cytoplasmic tail, would be expected to bind IgE. Furthermore, the chimaeric receptors which contained appropriate Fc ϵ RI or Fc γ RII sequences (e.g. as found in Chimaeras 4 and 5) would also be expected in a soluble form to bind IgG and/or IgE.
- 2) The second EC domain, EC domain 2, is intimately involved in the binding of IgE and IgG. Indeed, the binding of these immunoglobulins to Chimera 4 and Chimera 6 indicate that the amino acids that correspond to residues 87 to 128 inclusive of Fc ϵ RI are intimately involved with IgE binding and residues corresponding to amino acid residues 129 – 169 of Fc γ RII are intimately involved in IgG binding (Tables 3–5, Figure 8). These results identify these residues of the domain 2 as being important in the binding of IgE and IgG but do not exclude other regions of the receptor also being intimately involved.
- 3) It is clearly possible to construct multi-functional chimaeric Fc receptors that have properties of several different receptor types. Such multi-functional receptors would be expected to have advantages over individual receptor classes in that the production of a single chimaeric form of Fc receptor would have the functions of multiple Fc receptor classes and would circumvent the need for the production of two individual Fc receptor types with subsequent mixing of these receptors in any pharmaceutical preparation or assay system. The uses of these chimaeric receptors in the diagnosis and treatment of allergy, autoimmune disease, parasite infections, immune complex disease and a range of haemopoietic and non-haemopoietic disorders would be

significantly more straight forward, than using single function receptors.

4) Thus the manipulation of FcR will enable the construction of novel chimaeric FcR that can be high or low affinity and bind immunoglobulin of multiple classes. Receptors will have domain or subdomain sequences derived from different receptors (e.g. Ig binding domain or sequences of Fc ϵ RI or high affinity domains (D3) of Fc γ RII that are intimately involved in a particular functions resulting in a single receptor type having multiple functions). In addition, these functional regions could be attached to non FcR molecules e.g. antibody molecules, or bacterial proteins e.g. to create fusion proteins, wherein FcR function is maintained in addition to the function of the non FcR protein.

5) Furthermore, the monoclonal antibody experiments indicated that the EC domain structure is sufficiently conserved in some case eg. Chimaera 3, and not only interacts with Ig but retains the epitope detected by the monoclonal antibodies 8.7 and 7.30. However, some alteration to the total overall structure is apparent since the epitope detected by antibody 8.2 and 8.26 has been lost from Chimaera 3 as a result of the construction of the chimaeric receptors containing sequences from the Fc ϵ RI.

The studies described below of the characterisation of these monoclonal anti Fc γ R antibodies identify antibodies 8.2, 8.26, 8.7, 7.30 as unique monoclonal antibody which are embodied in this patent application.

The characterisation of the native and chimaeric FcR was aided by the use of monoclonal antibodies (MAb). These antibodies recognise cell surface Fc γ R and also soluble circulating FcR. This has been made possible by the production and characterisation of 4 new MAb (designated 8.2, 8.7, 8.26, 7.30) recognising human Fc γ RII is described and the use of these MAb in the detection of soluble Fc γ RII (sFc γ RII) in a two antibody radioimmunoassay.

Four MAbs were derived by cell fusion for characterisation. Three MAb [8.2 (of the IgG1 subclass), 8.7 (IgG1) and 8.26 (IgG2b)] were produced by immunisation of BALB/c mice with K562 cells. A fourth "second generation" MAb 7.30 (IgG1) was produced by immunisation with Fc γ RII 3.0 cDNA transfected L-cells. Cell fusions were performed as described in the Materials and Methods.

Tissue Distribution by FACS Analysis.

The tissue distribution of the antigens detected by the MAb was determined by quantitative analysis of fluorescence staining of Fc_YRII 3.0 transfected L-cells (Tf3.0) and a number of haemopoietic cell lines. This analysis shows that the four new MAb have strong specific positive reactions with Fc_YRII 3.0 cDNA transfected L-cells, similar to the two CDW32 reference MAb, IV.3 and CIKM5 described by others (22, 23) which are included for the purposes of comparison. The tissue distribution of the new MAb on the haemopoietic cell lines have profiles which distinguish these from each other and from IV.3 and CIKM5. All antibodies bound strongly to K562 cells but had unique binding on Daudi cells where only MAb 8.7 and 7.30 bound strongly to Daudi (Table 6). The MAb 8.26 bound less strongly and antibody 8.2 showed very weak binding as did the IV.3 and CIKM5 antibodies. Weak binding to Raji cells was also obtained with MAb 8.7, 8.26 and 7.30 and there was no detectable binding with the 8.2 antibody. All antibodies bound to an EBV transformed B cell line (Table 6). It should be noted that the MAb 8.26 gave consistently higher fluorescence on B cell lines (Daudi, Raji, EBV cells) than did the 8.2 and IV.3 MAb.

The binding to myeloid cells was also examined. Antibodies bound to the U937, Thp-1 and HL60 cell lines. However 8.7 and 8.26 could be distinguished from other MAb as they bound less strongly to U937 cells (Table 6). The fluorescence profile s of the binding of the MAb to Daudi and U937 cells is shown in Figure 9. Antibodies IV.3, CIKM5, 8.2 and 8.26 react with greater fluorescence intensity on U937 than on Daudi. Conversely antibodies 8.7 and 7.30 exhibit greater fluorescence than 8.2 or 8.26 or IV.3 and CIKM5. It should be noted that 8.26 has greater reactivity on all B cell lines and can be distinguished from 8.2, IV.3 and CIKM5 (Figure 9). All 6 MAb were negative on the 4 T-cell lines tested.

Inhibition of Fc Binding.

To determine the relationship of the epitopes detected by these antibodies to the Ig binding site, the capacity of these antibodies to inhibit immune complex (IgG-EA) binding was assessed (Table 7). Results of EA inhibition assays on three Fc_YRII

bearing cell lines is shown in Table 2. The three cell lines K562, Fc γ RII cDNA transfectants (Tf3.0) and Daudi cells express only Fc γ RII. The pattern of inhibition separates the 6 MAb into 3 groups; IV.3, 8.2 and 8.26 give 80-100% inhibition of EA rosetting on all 3 cell lines. The 8.7 and 7.30 antibodies completely inhibit EA rosetting on Daudi cells, but only partially inhibited EA rosetting on K562 and transfected L-cells. CIKM5 alone, gives partial inhibition of EA on K562 only.

Competitive Inhibition Assays.

To Determine Relationship Between Epitopes detected monoclonal antibodies. Competitive inhibition assays between the MAb were initially performed on K562 cells (Table 8) and on transfectants expressing Fc γ RII (Figure 10). When tested on the K562 cells radiolabelled IV.3, 8.2 and 8.26 show similar complete reciprocal inhibition patterns with unlabelled IV.3, 8.2 and 8.26 i.e. they completely inhibit each others binding indicating these MAb recognise overlapping epitopes. The epitope detected by the IV.3 antibody was unique, as unlabelled IV.3 (but not 8.2 and 8.26) completely inhibited the binding of labelled 8.7, 7.30 and CIKM5. Antibodies 8.7 and 7.30 formed a second distinct group as these completely inhibited each others binding to K562 cells indicating these detect identical epitopes. However, 8.2 or 8.26 did not inhibit the binding of 8.7 and 7.30. Unlabelled CIKM5 either failed to inhibit or only partially inhibited the binding of all other anti-Fc γ R MAb tested (Table 8). As discussed below, these results taken together with the tissue distributions and EA inhibition studies indicate the presence of at least 4 epitopes:- Epitope 1 detected by the IV.3 antibody. Epitope 2 detected by the CIKM5 antibody. Epitope 3 by the 8.2 and 8.26 antibodies and Epitope 4 detected by antibodies 7.30 and 8.7.

The competitive binding experiments were also performed on Fc γ RII cDNA transfected L-cells which express a single form of Fc γ RII (Figure 10). One MAb defining each of the 4 possible epitopes was studied (IV.3, CIKM5, 8.2, 7.30). The results of competitive inhibition assays are shown in Figure 10. The studies with the Fc RII transfected cells confirms the pattern of inhibition seen when K562 cells were used as target cells.

Immunoprecipitation.

The Fab or Fab'₂ fragments of the anti Fc γ RII MAb were used for

immunoprecipitation studies from cell lysates of surface labelled K562 cells or Daudi cells (Figure 11A,B). Antibodies IV.3, CIKM5, 8.2 and 8.26 precipitated protein from K562 cells which migrated as a broad band on an SDS-PAGE gel with a Mr of 40-43kd. No material was precipitated with 8.7 antibody. A similar 40-43kD was immunoprecipitated with IV.3, 8.2 and 8.26 MAb from Daudi cells. In addition, aggregated human IgG and a rabbit polyclonal anti-Fc γ RII antibody immuno precipitated material of identical Mr to that of the Fc γ RII precipitated by the monoclonal antibodies (Fig. 11A, B).

Assay for circulating soluble Fc γ RII.

Using a two antibody sandwich radioimmunoassay normal individuals and a series of patients with autoimmune disorders (Systemic Lupus Erythematosis [SLE], Rheumatoid arthritis and Sjogren Syndrome) were tested for serum levels of soluble Fc γ RII. The results of this assay indicate that soluble Fc γ RII may be detected in normal individuals and patients with autoimmune diseases have significantly higher circulating levels of soluble Fc γ RII. The presence of higher levels of circulating Fc γ RII is most evident in serum from individuals with Sjogren's Syndrome. Elevated levels were also detected in the circulation of patients with rheumatoid arthritis or Systemic Lupus erythematosis.

The production and characterization of four monoclonal antibodies to human Fc γ RII is useful in the study of cellular and circulating Fc γ RII and its role or presence in disease. The four new monoclonal antibodies (8.2, 8.26, 8.7, 7.30) clearly detect human Fc γ RII. Since they:-

- 1) Bind specifically to mouse L cells transfected with human Fc γ RII cDNA but do not bind to the untransfected cells.
- 2) Show a tissue distribution that is identical to that expected for Fc γ RII.
- 3) Immunoprecipitate a 40 - 43 kd molecule that is the expected size of Fc γ RII and is the subject of Australian Patent Serial Number 595623.
- 4) All antibodies partially or completely inhibit the binding of immune complexes (IgG- EA) to Fc γ RII positive target cells.

Clearly these antibodies detect Fc γ RII molecules. However it is clear that the antibodies fall into two discrete groups which when taken together with experiments

using two previously defined monoclonal Fc_YRII antibodies, indicate that at least four epitopes exist on Fc_YRII. The groups defined thus:-

- (i) 8.2, 8.26
- (ii) 8.7, 7.30
- (iii) IV.3
- (iv) CIKM5

These antibody groups can be distinguished from each other on the basis of (i) tissue distribution (ii) differences in the capacity to inhibit the binding of immune complexes, EA rosettes - (CIKM5 can only weakly inhibits EA rosetting whereas all other antibodies completely or significantly inhibit rosetting) and (iii) competitive inhibition assays indicate that the antibodies in the groups shown above detect four distinct epitopes. These distinct epitopes may be generated on mature cell surface Fc_YRII by post translation modification, such that the epitopes are carbohydrate determined; alternatively the antibodies detect protein epitopes in the peptide core.

It is clear that multiple forms of Fc_YRII have been defined (4-6, 8). However, these different forms have highly homologous extra cellular regions, wherein the amino acid identity was greater than 90% and the nucleic and amino acid sequence of the EC domains of the Fc_YRII, b1 and b3 are in fact identical. This high conservation of amino acid identify also results in high conservation of Fc_YRII function, in that all Fc receptors identified have the capacity to bind IgG immune complexes. To determine if the monoclonal antibodies described herein (8.2, 8.26, 8.7, 7.30) can distinguish between extracellular sequences of these multiple isoforms, transfection experiments were performed wherein cells were transfected with human Fc_YRII a clone 3.0 or transfected with the human Fc_YRII b1 clone (8). All monoclonal antibodies, 8.2, 8.26, 8.7, 7.30 bound to both Fc_YRIIa and Fc_YRII b1 and since the amino acid sequence of EC portion (both domains) of Fc RII b1, b2, b3 is identical (8) these MAb would detect Fc_YRIIb2 and b3 also.

Since the antibodies 8.26 and 8.7 detect distinct non overlapping epitopes, a capture tag radio immunoassay for the detection of soluble Fc receptor was developed. One monoclonal antibody was attached to the PVC plate and used to capture circulating Fc receptor in the blood of normal or diseased individuals. A

second antibody was used to detect the captured Fc receptor, this second antibody was tagged (eg. radio- labelled) on such a way that binding of this antibody could be detected. As shown in Figure 12 the second antibody was radiolabelled with I 125 and the specific binding of radiolabelled antibody to captured Fc receptor indicated the presence of circulating Fc receptor in blood derived from different individuals. In this study three groups of patients with autoimmune disease were studied: SLE, RA and Sjogrens syndrome. When compared to normal individuals all groups of patients with autoimmune disease has statistically significant high levels of soluble Fc γ RII. It is clear that this assay can be used to identify circulating soluble receptor and the assay can be adapted to be performed with other monoclonal antibodies or polyclonal receptor antibody. The second antibody can also be radiolabelled or conjugated to a flurochrome or enzyme and be used in photometric or colormetric assays, e.g. ELISA assays.

The detection of circulating Fc γ R may be of diagnostic use for patients with autoimmune or other diseases where high levels of receptor are of diagnostic or prognostic value or as an indicator of disease activity. Indeed, the patients afflicted by Sjorgens Syndrome and the diseases indicated (SLE and RA) (Figure 12) have much higher levels of circulating receptor than normal individuals. Although the source of the circulating Fc γ RII is unknown it is clear that it can be detected in the circulation;. Furthermore, as all the MAb - detect all isoforms of Fc γ RII - i.e. Fc γ RIIa, Fc γ RIIb1 (and Fc γ RIIb2, b3) then the assay would also detect the circulating forms of these. However, the fact that the precise nature of the circulating Fc γ RII is unknown and does not compromise the use and application of the assay for the detection of soluble Fc γ RII.

The application of this assay for the detection of soluble Fc receptor is not restricted to use in assays of patient serum but is equally applicable for assays using other bodily fluids, eg. urine, saliva, synovial fluid, or faeces.

Concluding Remarks

We have demonstrated the principle that chimeric Fc receptors derived in such a fashion that they contain amino acid sequence from multiple Fc receptors can also retain the immunoglobulin binding functions of these receptors types. Also the

identification of the function or active region of these receptors (ie. immunoglobulin interactive) regions also identifies a useful region of these molecules. Monoclonal antibodies were used to demonstrate the preservation of epitopes in these chimaeric molecules and detect soluble Fc receptor in the circulation of diseased individuals.

MATERIALS AND METHODS

Molecular genetic techniques.

Standard molecular genetic techniques were performed as described in references 13 and 14 and included; restriction digestion, electrophoresis and purification of DNA fragments, ligation, bacterial transformation, di-deoxy nucleotide sequencing, plasmid DNA preparation, nucleic acid phosphorylation and de-phosphorylation, hybridisation, Southern blots.

Splice overlap extension (SOE).

Chimaeric cDNA (which encode chimeric Fc receptors) were produced by splice overlap extension (SOE) using three polymerase chain reaction (PCR) steps essentially as described (24,25). The first PCR was performed to amplify sequence that forms 5' section of the chimaeric cDNA. The second PCR amplified sequence that forms the 3' section of the chimeric cDNA. Oligonucleotides (500ng) hybridising to the 5' or 3' ends of the region to be amplified were mixed with the appropriate template (100ng) and 25 cycles were performed using a thermal cycler (see below). The sequence of oligonucleotides used in the PCR reactions were designed such that the oligonucleotide primer hybridising to the 3' end of the first PCR product (this makes up the 5' sequences of the chimeric cDNA) overlaps sequence contained within the oligonucleotide primer hybridising to the 5' end of the second PCR product (this makes up the 3' sequences of the chimaeric cDNA) (Figures 6,7 Table 3,4,9). Thus the two independently derived PCR products overlap at their 3' (PCR product 1) and 5' (PCR product 2) ends.

The splice overlap extension reaction (ie third PCR) generates the chimeric cDNA for subsequent manipulation and was performed using purified PCR products 1 and 2. Approximately 10ng of each of the PCR product was mixed with two oligonucleotide primers (used in PCR 1 and PCR 2) that hybridise to the 5' end of PCR product 1 or the 3' end of PCR product 2. Also the 5' oligonucleotides (NRI or

EG6) used in the third PCR contained a EcoRI recognition sequence for subsequent subcloning into the pKC3 vector. Similarly oligonucleotide EG5 that hybridises to the 3' end of the chimeric cDNA contained a Sall recognition site for subsequent subcloning of the chimaeric cDNA into the pKC3 vector. The PCR reaction were conveniently performed under the standard conditions i.e. oligonucleotides and template (quantities as required - see above) were mixed with 2.5U of Taq DNA polymerase in 10mM Tris-HCl pH 8.3, 50mMKCl, 1.5MgCl₂ (Varied according to oligonucleotide primer combination). Twenty-five cycles were performed each cycle consisting of denaturation at 94°C for 1 minute, annealing for 2 minutes at appropriate temperature and extension at 72°C for 3 minutes.

Cloning of hFcεRI cDNA.

1st strand cDNA was produced from human PBL (26). PCR was performed on 1st strand cDNA using oligonucleotide primers MDH13 5'TTAGATCTCAGCACAGTAAGCACC 3' which hybridizes to nucleotides position 1 to 17 of FcεRI (non-coding strand) (12) and MDH14-5' TTTAGATCTAAATTGAACATCTCTTTAC 3' (positions 1042 TO 1062). Both oligonucleotides contain a Bgl-II sit at their 5' ends for subcloning of the PCR product into pKC3 vector. The nucleotide sequence of the cloned FcRI DNA was determined by dideoxy-nucleotide sequencing (13,14) and is shown in Figure 7.

FcγR cDNA.

Mouse FcγRII and FcγRI cDNA used herein have been previously described (4, 5, 7). The human FcγRIIa cDNA sequence used herein has been described (6, Australian Patent Serial No. 595623) and the sequence shown in Figure 6.

Production of Monoclonal Antibodies. BALB/c mice, 8-10 week females, were immunised by weekly intraperitoneal injection of whole cells (0.5mls of 10⁸-10 cells/ml) for a least 3 weeks with either the K562 cell line or L-cells transfected with the human FcγRII 3.0 cDNA (Tf3.0) (6). Cell fusions using the NS-1 myeloma cell line were performed as described (29). Hybridoma supernatants were screened for MAbs by two stage rosetting of target cells using sheep anti-mouse immunoglobulin coupled to SRBC via CrCl2 (28). Target cells used for screening were both K562 and Tf3.0 cell line. Positive hybridomas were then cloned by limiting dilution at least

twice and then grown as ascites tumors in (CBAxBALB/c)F1 mice primed with pristane. MAbs were purified from ascites using Protein A Sepharose chromatography. Other MAb used in the study included IV.3 (IgG2b) and CKM5 (IgG1) both standard anti CD32 MAb (22, 23). Anti-Ly2,1 antibody 49.11.1 (IgG2a) and 49-17.1 (IgG), and 5084-4.1 (IgG2a) anti-Ly-12.1 (IGg2A) used as a negative control antibodies.

Quantitative immunofluorescence.

Binding of MAb to haemopoietic cell lines was quantitated on a Facscan by immunofluorescence. 25ul of ascites or serum (1:400 dilution) or aggregated human IgG in PBS was added to target cells (5×10^6 cells/ml) in PBS-BSA 0.5% and incubated for 45 minutes on ice. The monocyte cell lines U937 and HL-60 were pre-incubated with 25 ul of 5mg/ml of human immunoglobulin to block non specific Fc binding to high affinity Fc γ RI. Cells were washed and resuspended in fluorescein isothiocyanate (FITC) sheep anti-mouse Ig F(ab)'2 (silenus) 1:50 dilution was then incubated on ice for 30 minutes in the dark. Cells were washed and resuspended in 0.5ml PBS-BSA 0.5%.

Inhibition of EA Rosetting.

The ability of the MAb to block Fc binding was determined by inhibition of rosetting with polyclonal rabbit antibody coated erythrocytes (EA). The inhibition assay was performed as follows: 50ul of the blocking antibody ascites or serum was serially diluted and incubated with 50ul of Fc γ R bearing target cells at a concentration of 5×10^6 /ml for 45 minutes on ice. Cells were washed free of excess antibody. 50ul of EA was added to the cell and spin at 200g for 4 minutes. The EA's and cells were incubated for 30 minutes on ice. Cells were stained with Ethyl Violet and a typical field of 100 cells was assessed for rosette formation (at least 5 RBC bound or 50% of cell surface covered). Target cells used were K562, Daudi and Tf3.0. Sensitisation of sheep erythrocytes with antibody was performed as described (5, 29).

Competitive inhibition Assays.

Purified antibodies (100ug) were labelled with carrier free I¹²⁵ using the Chloramine T method 29) but labelling performed for 30 seconds on ice with a

Chloramine T concentration of 1mg/ml. Free iodine was removed on a Sephadex PD-10 column (Pharmacia). Flexible microtitre plates were coated with 5% skim milk overnight to reduce non specific binding. Serial two-fold dilutions starting at a final concentration of 25ug/ml of unlabelled antibody (25ul) was mixed with 25ul of a fixed predetermined dilution of radiolabelled antibody. Finally 50ul of target cells 10^7 cells/ml) was added to the mixture of labelled and unlabelled antibody, and incubated at 4°C for 4 hours. Cells were washed and assayed for bound radioactivity. Value of radioactivity bound to cells in absence of unlabelled antibody was taken as 100% binding. The % inhibition was calculated as follows:-

$$\frac{(\text{max. cpm}) - (x\text{cpm})}{\text{max.cpm}} \times 100$$

$$\text{max.cpm} \quad 1$$

where x=cpm of assayed cells and max.cpm = 100% binding value. Target cells used were Fc_YRII cDNA transfected L-cells and K562 cell.

Unlabelled irrelevant antibodies 49-11.1 or 49.17.1 antibodies were used to assess non-specific binding.

Immunoprecipitation.

Cell suspensions (5×10^7 to 10^8) were surface labelled with carrier free I¹²⁵ using a modified lactoperoxidase method (29). I¹²⁵ (10ul of 1mCi/ml; Amersham) and lactoperoxidase (80ul, 1mg/ml PBS; Sigma) was added to the cell suspension and surface radioiodination initiated by sequential addition of H₂O₂ (BDH) (20ul) of 1:27,000, 1:900, 1:2000, 1:1000 dilution of 30% v/v solution in PBS) at 2 minute intervals. The reaction was stopped by addition of cold PBS and the cells washed twice in PBS. Cells were then solubilised in lysis buffer (0.01M Tris.Ha), 15M NaCl, 0.5% NP 40, 1mM EDTA and 1mM Phenyl sulphonylfluoride pH=8) at 4°C for 2 hours. Nuclei were removed by centrifugation at 10,000g for 10 min. Free iodine was removed on a Sephadex PD-10 column. Immunoprecipitaion was carried out by incubating 50ul antibody (Fab/Fab'2) conjugated sepharose beads (washed in lysis buffer) with 1ml of cell lysate at 4°C overnight. Polyclonal antibody or HAGG were incubated with 50ul of protein A conjugated Sepharose and then with 1ml of cell lysate at 4°C. The following day beads were washed in lysis mix, dissolved in SDS-PAGE sample buffer and analysed on a 10% SDS-PAGE gel under reducing

conditions (29). Dried gels were autoradiographed.

Assay for Human Soluble Fc_YRII Using a Two Antibody Sandwich Radioimmunoassay.

Two monoclonal antibodies (8.2 and 8.26) to human Fc RII detecting different epitopes were purified from ascites using Protein A Sepharose chromatography (29). Flexible 96 well PVC microtitre plates (Costa) are coated with the first antibody 8.26 using 50-70ul per well at a concentration of 10ug/ml of antibody diluted in 0.005M carbonate/bicarbonate coating buffer pH=9.6 and incubated for 2 hours at 37°C. The plates are then washed three times by flooding the plates in PBS/0.05% Tween 20. The remaining sites in the wells are then blocked by coating the wells with 200 ul of 5% BSA and incubating for 1 hour at 37°C to reduce non-specific binding. The plate is then washed three times in PBS/0.05% Tween 20. Serum samples are diluted 1:4 in 2% BSA and 50ul is added to each well and incubated at 4°C overnight. The plates are then washed three times in PBS/0.05% Tween 20. The second radiolabelled antibody (8.7) was then added. 50ul of ¹¹²⁵ labelled 8.7 (50x10⁴ cpm/50ul) is added to each well and incubated for 4 hours at 4°C. The plates were washed four times by flooding in PBS/0.05% Tween 20 and individual wells were cut and counted in Gamma counter.

Transfections.

Transfection of plasmid DNA into COS-7 cells was performed using DEAE dextran as described (10, 13, 14).

Monoclonal antibodies.

The monoclonal anti-Fc_YRII antibody IV.3 was obtained from Dr Clark Anderson (Ohio State university) and the CIKM5 antibody from Mr Glen Pilkington (Cancer Institute, Melbourne).

The IgG anti-TNP antibodies used have previously been detailed (5). The IgE anti-TNP antibody, TIB-142 was obtained from the ATCC (Maryland, USA). The monoclonal anti-glycophorin antibody was obtained from Dr Leonie Ashman (University of Adelaide).

The 2.4G2 antibody has been described (30).

Fab' or Fab fragments of antibodies were produced as described (29).

Polyclonal anti-Fc γ RII antibodies.

Rabbit antibody detecting human Fc γ RII was produced by immunisation of New Zealand White rabbits with purified Fc γ RII fusion protein. The rabbit were immunized at regular intervals five times 3-5mg of fusion protein. The first immunisation was performed intradermally in multiple sites in complete Freunds adjuvant and subsequent immunisations in incomplete Freunds adjuvant.

The bacterial fusion protein was produced using the pATH21 vector (31) by cloning the large Pst-1 - XbaI fragment of Fc γ RIIa cDNA (Figure 6) into the Pst1 site and XbaI sites of pATH21. The XbaI site being introduced by PCR mutagenesis into the Fc γ RLLa cDNA at position 627-632.

The induction and purification of the fusion protein by electrophoresis was performed as described (31)

Scatchard Analysis.

Scatchard analysis was performed as described (32) using monomeric mouse I¹²⁵ - IgG2a prepared by incubating 100ug of IgG2a (in 50ul of 0.3M phosphate buffered saline pH7.4) with 500uCi Na¹²⁵ (Amersham UK) in the presence of chloramine - T Merck) at 1mg/ml in PBS pH7.4, for 30 sec. on ice. Labelling reactions were stopped with 50ul of 2.4mg/ml sodium metabisulphite and 10mg/ml tyrosine; free ¹²⁵ was removed from labelled protein by passage through a PD-10 Sephadex G-25 column (Pharmacia, Uppsala, Sweden. COS cells transiently transfected with FcR cDNAs were harvested 48hr after transfection, washed twice in PBS containing 0.5% BSA and resuspended to 2x10⁶/ml in L15-0.05% BSA for use in Scatchard analysis. 50ul aliquots of cells were incubated with 50ul serial dilutions of ¹²⁵ - IgG2a in L15 medium for 60 min at 25°C with periodic agitation. Cells were then pelleted by centrifugation through a 3:2 (v/v) mixture of dibutylphthalate and dioctylphthalate oils (Fluka Chemika, Switzerland) and cell bound ¹²⁵ -IgG2a assayed. Parallel experiments were performed under identical conditions in the presence of 100 fold excess of unlabelled IgG2a to correct for non-specific Ig binding.

Oligonucleotides

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Oligonucleotides were synthesised as described (27) and are listed in Table 9.

The invention will be further described with reference to the following figures:

Figure 1

Mutagenesis of Fc γ RI. PCR was employed to introduce an Apa I site between D2 and D3 of Fc γ RI cDNA to facilitate domain exchange with Fc γ RII cDNA. Two PCR reactions were performed, one to produce a fragment containing D1 and D2 coding regions and a second PCR to produce a fragment containing D3, the transmembrane and cytoplasmic tail coding regions. Each of the fragments were engineered to have an Apa I site introduced into the Fc γ RI sequence between the D2 and D3 coding regions – through the use of overlapping and partly complementary oligonucleotide PCR primers (the Apa I site is indicated by solid circles). Each PCR reaction also employed an oligonucleotide primer containing a Sal I site (solid box), thus producing Fc γ RI DNA fragments containing Sal I and Apa I sticky ends suitable for construction of the chimeric receptor cDNAs (see Fig. 2).

PCR conditions: The complementary oligonucleotide designed to introduce the Apa I site had the following sequences:

MDH1 5' GGTGAA GGGCCC TTTCACCGTGATGG 3'

MDH2 GGTGAAAGGGCCC TTCACCACGCCAG 3'

MDH1 corresponds to nucleotides 573 to 599 and MDH2 corresponds to nucleotide 580 to 605 of the Fc γ RI cDNA sequence.

Boxed sequence contains the Apa I site.

The oligonucleotides containing the Sal I site were as follows:

MDH3 5' TTT GTCGAC ATGATTCTTACCA^{GC}CTTGGAGATG 3'

MDH4 5' TTT GTCGAC CCCCGGGGATCCTCTAGAGTCGAC 3'

The boxed sequence contains the Sal I site. MDH3 hybridizes to Fc γ RI 5' untranslated sequence from position 1–26 the first 8 bases of MDH3 contain 3 spacer nucleotides and 5 bases of the Sal I sequence.

MDH4 hybridizes to the pGEXII vector sequence flanking the 3' end of Fc γ RI cDNA insert.

The two PCR employed the oligonucleotide pairs MDH1 and MDH3 to produce the D1 and D2 fragment and MDH2 with MDH4 to produce the D3, tm and cytoplasmic tail coding fragment. PCRs were performed under the following

conditions: 1ng of Fc γ RI cDNA (cloned in the vector pGEXII) was used as a template for amplification of the two fragments outlined above. 25 cycles were performed using a Perkin-Elmer-Cetus DNA thermal cycle in the presence of 500ng of each oligonucleotide primer, 10mM Tris-C1 ph 8.3, 50mM KC1, 1.5mM mgCl₂, and 2.5 units of taq polymerase (AmpliTaq - Perkin - Elmer - Cetus). The PCR products were extracted with phenol: chloroform and ethanol precipitated. The precipitates were dried, dissolved in buffer and digested with Sal I and Apa I, extracted, precipitated and used in subsequent cloning steps see Fig. 2.

Figure 2

Construction of expressable chimeric FcR cDNA's.

Chimeric cDNA's were generated by replacing Fc γ RII coding domains in the expression vectors with Fc γ RII coding domains generated by PCR. (A) Fc γ RII cDNA was subcloned into the Pst I site of the expression vectors pKC3 and pKC4 (derived from the parental vector pKO-neo Van Doren, et al J. Virol. 50 (1984) in expressable orientation [pKC3 and pKC4 differ only in the orientation of the polylinker polycloning sites such that the Sal I site flanks the 5' or 3' end of the cDNA insert in pKC3 or pKC4 respectively].

(B) To produce the Fc γ RI-II chimera (consisting of D1 and D2 of Fc γ RII linked to the transmembrane and cytoplasmic coding regions of Fc γ RII) D1 and D2 of Fc γ RII were removed from pKC3-Fc γ RII by digestion with Sal I and Apa I and (C) replaced with a Sal I Apa I fragment containing D1 and D2 of Fc γ RII derived by PCR (see figure 1).

(D) Similarly, for generation of the Fc γ RII-I chimera (consisting of d1 and d2 of Fc γ RII linked to D3, the transmembrane and cytoplasmic coding domains of Fc γ RI) the cDNA sequences encoding Fc γ RII transmembrane and cytoplasmic regions were removed by digestion with Sal I and Apa I, and (E) replaced with Sal I - Apa I fragment containing D3, the transmembrane and cytoplasmic coding domains of Fc γ RII derived by PCR (see Figure 1).

Figure 3

Nucleotide and amino acid sequence of chimeric Fc receptors. Nucleotide positions are numbered in decades below the line in a 5' to 3' direction. Untranslated

sequence is shown in closed type.

- A. Chimeric Fc γ RI/II. Fc RI derived cDNA sequence from positions 9 to 594, Fc γ RII from 595 to 1244.
- B. Chimeric Fc γ RII/I. Fc γ RII derived cDNA sequence from positions 1 to 662, Fc γ RI from 663 to 1348 between Fc γ RI and Fc γ RII domains. The Apa I restriction site GGGCCC in both chimeric sequences is underlined. Sal I sites introduced into Fc γ RI sequence by PCR to facilitate cloning are boxed.

Figure 4

IgG immune complex binding to chimaeric Fc γ R using antibody sensitized erythrocytes (EA). COS-7 cells were transfected with cDNA encoding Fc γ RI (A,F,G); Fc γ RII (D); (Fc γ RI/II (C,I,J); Fc γ RII/I (B); vector only (E,H). The transfected COS cell monolayers were tested for Fc binding using erythrocytes sensitised with antibody. Rabbit IgG-EA were used in (A-E) and mouse IgG1-EA (G,H,J) and mouse IgG2a-EA (F,I). Methods described in Materials and Methods Section.

Figure 5

Scatchard analysis of IgG2a binding to Fc γ RI transfected COS-7 cells. Data has been subjected to linear regression analysis.

Figure 6

Nucleotide and deduced amino acid sequence of human Fc γ RIIa (6). Amino acid positions are numbered above the line in decades commencing at the proposed amino acid terminal end. (Signal sequence is numbered from residue -34 to -1). Nucleotide positions are numbered at the end of the line. Oligonucleotide primers used in the PCR reactions for construction of the chimaeric cDNA are positioned at their priming sites with 5' to 3' direction indicated by half arrow heads. Solid lines represent oligonucleotide sequence derived from the Fc γ RII cDNA template, dotted lines represent oligonucleotide sequence derived from Fc ϵ RI cDNA template (Figure 7). Solid boxes represent 5' terminal EcoRI sites, solid circles 5' terminal SalI sites.

Figure 7

Nucleotide and deduced amino acid sequence of human Fc ϵ RI. Amino acid positions are numbered above the line in decades commencing at the proposed amino

acid terminal end (signal sequence is numbered from residue -25 to -1). Nucleotide positions are numbered at the end of the line. Oligonucleotide primers used in the PCR reactions for constructions of the chimaeric cDNA are positioned at their priming site with 5' to 3' direction indicated by half arrow heads. Dotted lines represent oligonucleotides sequence derived from the Fc ϵ RI cDNA template, solid line represents oligonucleotide sequence derived from Fc γ RII cDNA template (Figure 6). Solid boxes represent 5' terminal EcoRI sites.

Figure 8

Rosetting of COS-7 cells transfected with FcR cDNA. COS 7 cell monolayers were transfected with human Fc γ RII cDNA (A,B); Chimaera 1 cDNA (C,D); Chimaera 2(E,F); Chimaera 3 (G,H); chimaera 4 (I,J). Seventy-two hours later the binding of antibody sensitised erythrocytes was assessed using IgE-EA (A,C,E,G,I) or IgG-EA (B,D,F,H,J).

Figure 9

Fluorescence histograms of immunofluorescence of anti-Fc γ RII monoclonal antibody staining of Daudi cells of U937 cells as indicated. Horizontal axis represents fluorescence intensity (log scale) the vertical axis represents relative cell number.

Figure 10

Competitive binding of unlabelled monoclonal anti-Fc RII antibodies. Competitive assay performed using L-cells transfected with human Fc γ RII cDNA HFc 3.0 Radiolabelled antibodies are (A), IV.3; (B), CIKM5; (C), 8.2; (D), 7.30. The cold competitors are shown, Ly-2.1 (49-11.1); IV.3; CIKM5; 8.2; 7.30.

Figure 11

Immunoprecipitation of Fc γ RII from surface labelled K562,(A) or Daudi,(B) cells. Molecular weight standards (Mr \times 10, (Std) are indicated on the left side of the figure. Antibodies (Fab or Fab'2 fragments) used are IV.3, CIKM5, 8.2, 8.7, 8.26. Human aggregated IgG (HAGG) intact Ly-12.1; 1705(5084-4.1). The negative control for the polyclonal antibody was immunoprecipitation from Fc γ RII-CEM cells.

Figure 12

Detection of circulating Fc_YRII in the serum of normal or diseased individuals. (A) mean cpm \pm 1 S.E. of bound anti-Fc RII in the patient groups indicated. P values are calculated for comparison of patient groups and normals. The numbers (n) of individuals tested within each patient group are indicated. (B) Dot plot of data obtained for individual patients within each group. Each point represents number of cpm (labelled anti-Fc RII antibody) bound.

The entire contents of the provisional specification lodged with Australian Patent Application of which this is the complete specification is hereby imported into this specification and forms part of the disclosure of this specification. The claims form part of the disclosure of this specification.

DEFINITIONS:**Extra functional region:-**

Part of a molecule, having function other than immunoglobulin binding.

Functional region:-

Part of molecule capable of interacting with immunoglobulins or their fragments whether the immunoglobulins or fragments are monomeric in nature, aggregated or immune complexes.

Immunoglobulin binding molecule:-

Any molecule capable of interacting with immunoglobulins or their fragments whether the immunoglobulins or fragments are monomeric in nature, aggregated or immune complexes.

Functional equivalents thereof:-

Variants on the materials the subject of this specification are possible:-

A. Sequence Variation

The nucleotide sequences encoding the receptor can be variable:-

1. Because of the degeneracy of the genetic code nucleotide change does not necessarily bring about a change in the amino acid encoded.
2. Two or three nucleotide changes can give rise to the same amino acid.
3. Changing one or two nucleotides may result in a conservative amino acid change unlikely to greatly affect the function of the protein.
4. Allelic variations. Variations in nucleotide sequence and resultant amino acid sequences of the encoded protein may occur between individual members of the same species. These variations arise from changes in the nucleotide sequences encoding the protein. Thus different forms of the same gene (called alleles) give rise to protein of slightly different amino acid sequence but still have the same function.
5. Proteins having the same function, e.g. immunoglobulin binding, may arise from related genes. Many protein gene families have been described.
6. Variation may be intentionally introduced by:-
 - (a) Mutating cloned cDNA or genomic DNA by point mutation, rearrangement or insertion of related or unrelated DNA into the cDNA or genomic clones

encoding the functional protein. Such mutated (variant) clones can be used to generate variant proteins or peptides.

- (b) By enzymatic cleavage of the protein (from either in vitro synthesis or normal cell synthesised protein) with or without repair/rearrangement of the cleavage products.
- (c) By chemical modification.
- (d) By irradiation.

Greek symbols:-

α alpha

γ gamma

δ delta

ϵ epsilon

μ mu

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TABLE 1

Binding of IgG subclasses to chimeric receptors

Transfecting DNA	Binding of IgG*					
	Mouse IgG subclasses ^t					
	<u>Human</u> IgG**	<u>Rabbit</u> IgG ^t	G1	G2a	G2b	G3
FcRI	+	+	-	+	-	-
FcRII	+	+	+	+	+	+
FcRI-III	+	+	+	+	+	+
FcRIII-I	+	+	+	+	+	+
NIL	-	-	-	-	-	-

* Ig binding assessed by EA rosetting for rabbit and mouse IgG. And

^t Ig binding level where >10 IgG coated erythrocytes bound per adherent Cos cell.

** binding assessed by immunofluorescence using heat aggregated-human poly clonal IgG and quantified by FACS analysis.

Erythrocyte - antibody complexes were prepared as follows:

- (i) Rabbit IgG sensitised erythrocytes were prepared as in Figure 1.
- (ii) EA coated with specific mouse IgG subclasses were produced by using anti-TNP isotype specific monoclonal antibodies (of the IgG1, IgG2a, IgG2b subclasses). Sheep red blood cells were sensitised with TNP by incubating a 10% SRBC suspension with 7 volumes of TNP in PBS (Phosphate buffered saline) for 20 minutes at room

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temperature. Sensitised cells were then washed twice in PBS+0.5% BSA and resuspended to - a 2% suspension upon which an equal volume of antibody was added and incubated for 60 minutes at room temperature.

(iii) Human red blood cells directly sensitised with IgG1 or IgG3 anti-glycophorin monoclonal antibodies as described above. EA's were used in rosetting assay again as outlined in Figure 4 legend.

Assays were performed directly in culture dishes on COS - 7 cells transiently transfected with FcR expression constructs (Fc RI in the pGEXII vector (Ref), Fc RII in pKC3, Fc RII-I in pKC4, Fc RII in pKC3), as described EA's were then washed twice in PBS-0.5% BSA again resuspended to a 2% suspension for use in rosetting following an approach outlined in Figure 3 legend.

Immunofluorescence was performed on transiently transfected cells. Polyclonal human IgG (5 mg/ml) was aggregated at 65°C for 30 minutes. Binding and immunofluorescence studies were performed as described (29).

TABLE 2

Detection 2.4G2 epitope on chimeric receptors
by blocking of EA rosetting

Transfecting DNA	% Blocking EA rosetting*
FcRI	0
FcRII	80
FcRI-RII	80
FcRII-I	80
Vector only **	0

* % blocking relative to transfected cells not treated with 2.4G2 antibody.

** Cos cells were transfected with pKC4 vector DNA.

Blocking assay were performed on Cos 7 cells transiently transfected with FcR expression constructs (see Table 1). Transfected cells in culture dishes were incubated whilst adhered with Fab fragments of the anti-Fc RII monoclonal antibody 2.4G2 (Unkeless; J.C. Exp. Med 150: 580-596, 1979) for 60' at 4°C. Cells (in dishes) were then washed x2 with L-15-0.5% BSA medium, and rabbit EA added to dishes. Rosette formation was assessed as outlined to Figure 3 legend.

TABLE 3 FcR cDNA AND CHIMAERIC FcR cDNA IN THIS STUDY

CHIMAERIC NAME	ORIGIN OF DOMAIN*			RECEPTOR COMPOSITION**			ORIGIN OF AMINO ACID RESIDUES†
	D1	D2	TM/CT	D1	D2	TM/CT	
FcεRI	ε	ε	ε				
FcγRII	γ	γ	γ				
1	ε	ε	γ				[ε(-25 → 169), γ (170 → 281)]
2	γ	ε	γ				[γ(-34 → 86), ε (87 → 169), γ(170 → 281)]
3	ε	γ	γ				[ε(-25 → 86), γ (87 → 281)]
4	ε	ε/γ	γ				[ε(-25 → 128), γ (129 → 281)]
5	ε	γ/ε	γ				[ε(-25 → 86), γ (87 → 128), ε(129 → 169), γ(170-281)]
6	γ	ε/γ	γ				[γ(-34 → 86), ε(87 → 128), γ(129 → 281)]
7	γ	γ/ε	γ				[γ(34 → 128), ε (129 → 169), γ (170 → 281)]

* D1, EC domain 1; D2, EC domain 2; TM, transmembrane; CT, cytoplasmic tail, ε, derived from FcεRI; γ, derived from FcγRII. Diagrammatic representation of origin of the regions of the Fc receptors. Unshaded regions derived from FcεRI; shaded regions derived from FcγRII. Origin of leader sequences not shown. The nucleotide and amino acid sequence of all native and chimaeric FcR was determined by dideoxy nucleotide sequence analysis (12, 13). Amino acid residues of the receptors. Numbering indicates the amino acids derived from FcεRI (ε) or FcγRII (γ) and numbers correspond to amino acid residues in Figures 6 and 7.

†

TABLE 4 CONSTRUCTION OF CHIMAERIC cDNAs

CHIMAERA NAME	ORIGIN OF DOMAINS			OLIGONUCLEOTIDE Pair I (Template)*	OLIGONUCLEOTIDE Pair II (Template)*
	D1	D2	TM/CT		
Fc γ RII	γ	γ	γ	-	-
Fc ϵ RI	ϵ	ϵ	ϵ	<u>EG6</u> + EG1 (Fc ϵ RI)	EG2 + <u>EG5</u> (Fc γ RII)
1	ϵ	ϵ	γ	<u>NR1</u> + EG11 (Fc γ RII)	EG10 + <u>EG5</u> (Chimaera 1)
2	γ	ϵ	γ	<u>EG6</u> + EG9 (Fc ϵ RI)	EG8 + <u>EG5</u> (Fc γ RII)
3	ϵ	γ	γ	<u>EG6</u> + EG14 (Fc ϵ RI)	EG15 + <u>EG5</u> (Fc γ RII)
4	ϵ	ϵ/γ	γ	<u>EG6</u> + EG12 (Chimaera 3)	EG13 + <u>EG5</u> (Chimaera 1)
5	ϵ/ϵ	γ	γ	<u>NR1</u> + EG14 (Chimaera 2)	EG15 + <u>EG5</u> (Fc γ RII)
6	γ	ϵ/γ	γ	<u>NR1</u> + EG12 (Fc γ RII)	EG13 + <u>EG5</u> (Chimaera 1)
7	γ	γ/ϵ	γ	-	-

* Underlined oligonucleotides used in SOE reaction; templates for PCR reactions shown in parentheses.

TABLE 5 PROPERTIES OF NATIVE Fc γ RII, Fc ϵ RI AND CHIMAERIC Fc ϵ R

CHIMAERA NAME	ORIGIN OF DOMAIN*			IMMUNE COMPLEX BINDING**								ANTI Fc ϵ RII MoAb BINDING†
	D1	D2	TM/CT	IgG	IgE	8.7	7.30	8.2	8.26	IV-3	CIKM5	
Fc ϵ RI	ϵ	ϵ	ϵ	-	-	-	-	-	-	-	-	-
Fc γ RII	γ	γ	γ	+	-	+	+	+	+	+	+	+
1	ϵ	ϵ	γ	-	+	-	-	-	-	-	-	-
2	γ	ϵ	ϵ	-	+	-	-	-	-	-	-	-
3	ϵ	γ	γ	+	-	+	+	-	-	+	-	-
4	ϵ	ϵ/γ	γ	+	+	ND	ND	ND	ND	ND	ND	ND
5	ϵ	ϵ/γ	γ	-	-	ND	ND	ND	ND	ND	ND	ND
6	γ	ϵ/γ	γ	+	+	ND	ND	ND	ND	ND	ND	ND
7	γ	γ/ϵ	γ	-	-	ND	ND	ND	ND	ND	ND	ND

* D1, EC domain 1; D2, EC domain 2; TM, transmembrane; CT, cytoplasmic tail; ϵ , derived from Fc ϵ RI; γ , derived from Fc γ RII.

** Immune complex binding assessed by EA rosetting using IgG and IgE sensitised erythrocytes. + = formation of rosettes; - = no rosette formation.

† Binding of monoclonal anti Fc γ R antibodies (MoAb) to cells transfected with native or chimaeric Fc receptors. Binding assessed by immunofluorescence and visualisation by fluorescence microscopy. +, fluorescent staining; -, no staining; ND, not determined.

TABLE 6 CHARACTERISATION AND TISSUE DISTRIBUTION OF MONOCLONAL ANTIBODIES

MoAb	Immunogen	Ig Class	Target Cells										
			Tf3.0	L-cells	K562	DAUDI	RAJI	EBV	U937	ThP1	HL-60	CEM	JURKATT
B cells													
IV.3	K562	γ2b	+++	-	+++	+	-	+	+++	+++	+	-	-
CIKM5	K562	γ1	+++	-	+++	+	+	+++	+++	++	+	-	-
8.2	K562	γ1	+++	-	+++	+	-	+	+++	+++	+	-	-
8.7	K562	γ1	+++	-	+++	+	+	+	++	++	+	-	-
8.26	K562	γ2b	+++	-	+++	+	+	+	+++	+++	+	-	-
7.30	Tf3.0	γ1	+++	-	+++	+	+	+	++	++	+	-	-

% reactivity with haemopoietic cell lines and human FcγRII transfected L cells compared to the negative control mAb 49.11.1.
 Ascites or serum was used at dilution of 1:400 0-10% reactivity = Negative (-); 10-50% reactivity = +; 50-75% reactivity = ++; 75-100% reactivity = +++. The control mAb 49.11.1 was negative in all cases.

Table 7

INHIBITION OF IMMUNE COMPLEX BINDING (EA-ROSETTING)

mAb	Target Cell		
	K562	Tf3.0	DAUDI
IV.3	100*	100	100
CIKM5	44	0	0
8.2	100	100	100
8.7	78	88	100
8.26	100	100	100
7.30	44	75	100
49.11.1	0	0	0

* Percentage of inhibition of EA rosette formation using all indicated antibodies at a 1:2000 final concentration of ascites or serum. Target cells indicated.

TABLE 8

COMPETITIVE BINDING ASSAY*

		RADIOLABELLED ANTIBODY					
Cold		IV.3	8.2	8.26	CIKM5	8.7	7.30
Competitor							
IV.3	+	+	+	+	+	+	+
8.2	+	+	+	+	-	-	-
8.26	+	+	+	+	-	-	-
CIKM5	±	±	±	+	±	-	-
8.7	±	-	-	±	+	+	+
7.30	±	-	-	±	+	+	+

* K562 is the target cell

+: % inhibition equivalent to that obtained with the same unlabelled antibody

e.g. unlabelled IV.3 inhibition of labelled IV.3.

±: partial or minimal inhibition of radiolabelled antibody

-: no inhibition of radiolabelled antibody.

TABLE 9 OLIGONUCLEOTIDES USED IN THE GENERATION OF CHIMAERIC FcR

NAME*	SEQUENCE 5' - 3'	NUCLEOTIDE POSITION ON FcεRI/FcγRII cDNA** (5' to 3' of oligonucleotide)
EG1	<u>5</u> GGGCACTGTACAGTAATGTTGAGGG <u>3'</u>	FcγRII 628-621; FcεRI 621-604.
EG2	<u>5</u> CATTACTGTACAAGTGCCAAGCATG <u>3'</u>	FcεRI 612-621; FcγRII 621 to 635.
EG5†	<u>5</u> TTTGTCGACCACATGGCATAACG <u>3'</u>	FcγRII 981-967
EG6†	<u>5</u> TTTGAATTCAAGCACAGTAAGCACC <u>3'</u>	FcεRI 6-22
EG8	<u>5</u> TTCAGTGACTGGCTGGTGCCTCAG <u>3'</u>	FcεRI 364-372; FcγRII 372-386
EG9	<u>5</u> ACCAGCCAGTCACTGAAGACTTCC <u>3'</u>	FcγRII 379-372; FcεRI 372-357
EG10	<u>5</u> CTTTCCGAATGGCTGCTCCTTCAG <u>3'</u>	FcγRII 363-371; FcεRI 373-387
EG11	<u>5</u> AGCAGCCATTGGAAAGCACAGTC <u>3'</u>	FcεRI 380-373; FcγRII 371-356
EG12	<u>5</u> TACCAAGTATTTCTGGATTTCACATT <u>3'</u>	FcεRI 506-499; FcγRII 497-479
EG13	<u>5</u> TCCCAGAAATACTGGTATGAGAACAC <u>3'</u>	FcγRII 489-497; FcεRI 499-516
EG14	<u>5</u> CGGGAGAACTTGGAGCTTCACCATC <u>3'</u>	FcγRII 505-498; FcεRI 498-481
EG15	<u>5</u> CTCTCAAGTTCTCCGGTTGATCC <u>3'</u>	FcεRI 491-498; FcγRII 498-514
NR1†	<u>5</u> TACGAATTCTATGGAGACCAATGTCTC <u>3'</u>	FcγRII 10-30

* Underlined oligonucleotides complementary to coding strand

** Sequence numbers taken from Figures 6, 7.

† These oligodeoxynucleotides also contain non homologous sequence including restriction enzyme recognition sequences.

CLAIMS:

1. A hybrid immunoglobulin binding molecule capable of binding to any one class or a plurality of classes of antibody molecules.
2. A hybrid immunoglobulin binding molecule according to claim 1 which is derived from any Fc receptor (FcR).
3. A hybrid Fc receptor molecule according to claim 2 comprising one or more functional regions of a first FcR linked to one or more functional regions of a second FcR or consecutive FcR.
4. A hybrid Fc receptor molecule comprising a polymer or a concatamer of heterogenous or homogenous functional regions.
5. A hybrid FcR according to any one of claims 1 to 4 wherein said functional regions are derived from different FcR molecules.
6. A hybrid FcR according to any one of claims 1 to 5 wherein said functional regions are derived from bacterial, mammalian or other origins.
7. A hybrid FcR according to any one of claims 1 to 6 wherein said functional regions are derived from human, mouse or a combination of human and mouse origins.
8. A hybrid FcR according to any one of claims 1 to 7 which is capable of binding any one of, or a plurality of antibodies selected from the classes IgG, IgM, IgA, IgD and IgE.
9. A hybrid FcR according to any one of claims 1 to 8 wherein said functional regions are derived from any one of FcR, Fc γ R, Fc γ R, Fc α R, Fc ϵ R, Fc μ R or IgE binding proteins.
10. A hybrid FcR according to any one of claims 1 to 9 wherein the said functional regions are derived from Fc γ R or Fc ϵ R being any one or a combination of the extracellular domains, or parts thereof, of Fc γ RI, Fc γ RII, Fc γ RIII, Fc ϵ RI, Fc ϵ RII or homologues or functional parts thereof.
11. A hybrid FcR according to any one of claims 1 to 10 wherein said functional domains comprise one or more extracellular domains, or parts thereof, derived from Fc γ RII of mammalian origin in combination with any one extracellular domain derived from the following:-

mammalian Fc γ R
mammalian Fc ϵ RI,
mammalian Fc ϵ RII,
mammalian Fc γ RI,
mammalian Fc γ RIII,
mammalian Fc α R,
mammalian Fc μ R, or
mammalian IgE binding proteins

12. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise the extracellular domain or parts thereof derived from Fc γ RI being amino acids 1-171 linked to the transmembrane region and cytoplasmic tail of Fc γ RII being amino acids.
13. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise extracellular domains or parts thereof, derived from Fc γ RII being amino acids 1-174 linked to the third extracellular domain, transmembrane region and cytoplasmic tail of Fc γ RI being amino acids 174-380.
14. A hybrid FcR according to any one of claims 1 to 11 wherein said functional region comprise an extracellular domain, or parts thereof, having greater than 90% homology to Fc γ RII or Fc ϵ RI of human or mouse origin linked to an extracellular domain of Fc ϵ RI of human or mouse origin.
15. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise an extracellular domain, or parts thereof, having greater than 50% homology to Fc γ RII or Fc ϵ RI of human or mouse origin linked to an extracellular domain of Fc ϵ RI of human or mouse origin.
16. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise an extracellular domain, or parts thereof, having greater than 35% homology to Fc γ RII or Fc ϵ RI of human or mouse origin linked to an extracellular domain of Fc ϵ RI of human or mouse origin.
17. A hybrid FcR according to any one of claims 1 to 11 wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from Fc γ RII having the following amino acid sequence :-

- Phe Ser Arg Leu Asp Pro Thr Phe Ser Thr Pro Gln Ala Asn His Ser His Ser Gly Asp Tyr His Cys Thr Gly Asn Ile Gly Tyr Thr Leu Phe Ser Ser Lys Pro Val Thr Ile Thr Val, or functional equivalent thereof.

18. A hybrid FcR according to any one of claims 1 to 11 wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from Fc ϵ RI having the following amino acid sequence : -

- Trp Leu Leu Leu Gln Ala Ser Ala Glu Val Val Met Glu Gly Gln Pro Leu Phe Leu Arg Cys His Gly Trp Arg Asn Trp Asp Val Tyr Lys Val Ile Tyr Tyr Lys Asp Gly Glu Ala Leu Lys, or functional equivalent thereof.

19. A hybrid FcR according to any one of claims 1 to 18 wherein said molecule is not bound to a membrane and is soluble in physiological and or non physiological buffers.

20. A hybrid FcR according to any one of claims 1 to 18 wherein said molecule is linked to any transmembrane region and/or cytoplasmic tail.

21. A hybrid FcR according to claim 20 wherein said transmembrane region and cytoplasmic tail are derived from FcR.

22. A hybrid FcR according to any one of claims 1 to 121 being a chimera comprising a first extracellular domain of Fc γ RII, a second extracellular domain of Fc ϵ RI and a transmembrane region and cytoplasmic tail of Fc γ RII.

23. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domains of Fc ϵ RI containing amino acids 1 - 169 of Fc ϵ RI linked to amino acids 170-281 of Fc γ RII.

24. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of Fc ϵ RI being amino acids 1-86 linked to a second extracellular of Fc γ RII being amino acids 87-169 linked to a transmembrane region and cytoplasmic tail of Fc γ RII being amino acids 170-281.

25. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of Fc ϵ RI containing amino acids 1-86 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 87-128 linked to part of the second extracellular domain of Fc γ RII being amino acids 129-169

linked to a transmembrane region and cytoplasmic tail of Fc γ RII being amino acids 170-281.

26. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of Fc ϵ RI being amino acids 1-86 linked to part of the second extracellular domain of Fc γ RII being amino acids 87-128 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 129-169 linked to a transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281.

27. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of Fc γ RII being amino acids 1-86 linked to part of the second extracellular domain of Fc γ RII being amino acids 87-128 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281.

28. A hybrid FcR according to any one of claims 1-21 being a chimera comprising the first extracellular domain of Fc γ RII being amino acids 1-86 linked to the second extracellular domain of Fc ϵ RI being amino acids 87-169 linked to a transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281.

29. A hybrid FcR according to any one of claims 1-21 being a chimaera comprising the first extracellular domain of Fc γ RII being amino acids 1-86 linked to part of the second extracellular domain of Fc ϵ RI being amino acids 87-128 linked to part of the second extracellular domain of Fc γ RII being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of Fc γ RII being amino acids 170-281.

30. A hybrid FcR according to any one of claims 1 to 29 further comprising one or more extra functional regions of FcR as hereinbefore defined.

31. The amino acid sequence comprising the hybrid FcR according to any one of claims 1 to 31.

32. A nucleotide sequence capable of encoding an amino acid sequence according to claim 31.

33. The nucleotide sequence of claim 32 comprising a cDNA or genomic DNA clone capable of encoding the amino acid sequence according to claim 31.

34. A method of producing hybrid immunoglobulin binding molecules

according to any one of claims 1 to 31 comprising the steps of linking different functional regions of different FcR molecules to construct a hybrid immunoglobulin binding molecule.

35. A method according to claim 34 comprising ligating a first FcR molecule at the transmembrane junction, ligating a second FcR molecule to obtain a suitable functional or non-functional region and linking said components.

36. A polyclonal antibody which reacts with any one of the hybrid FcR molecules as defined in claims 2 to 31, or parts thereof.

37. A monoclonal antibody which reacts with any one of the hybrid FcR molecules as defined in claims 2 to 31, or parts thereof.

38. A monoclonal antibody being of subclass IgG1 designated 8.2 as hereinbefore defined.

39. A monoclonal antibody being of subclass IgG1 designated 8.7 as hereinbefore defined.

40. A monoclonal antibody being of subclass IgG2b designated 8.26 as hereinbefore defined.

41. A monoclonal antibody being of subclass IgG1 designated 7.30 as hereinbefore defined.

42. Antibodies according to any one of claims 36 to 41 which detect a single epitope specific for FcR.

43. A biological product comprising one or more hybrid FcRs according to any one of claims 2 to 31 or antibodies thereto according to any one of claims 36 to 41 for interaction with a hybrid FcR as defined in any one of claims 2 to 31.

44. A biological product according to claim 43 wherein said interaction comprises any one or a combination of binding, detection, linking, inhibiting function.

45. A biological product according to any one of claims 43 or 44 which is capable of being bound to fixed solid support being a tube, dipstick, multiwell plate or other form made from PVC or other material.

46. A biological product according to any one of claims 43 or 44 which is capable of being bound to biologically active materials including cytokines,

hormones, red blood cells, liposomes, dialysis membranes, etc.

47. A biological product according to any one of claims 43 or 46 which is capable of being labelled with any one of labelling enzymes, radioactive isotopes, metal or non-metal particles, fluorescent molecules, free radicals chemiluminescent or bioluminescent molecules.

48. A biological product according to any one of claim 43 to 47 further comprising anti FcR antibodies or fragments thereof.

49. A diagnostic test kit comprising the biological product of any one of claims 43 to 48.

50. An assay for the FcR being an immunoassay utilizing the antibody or biological product of any one of claims 36 to 48.

51. An assay according to claim 50 being a capture tag assay.

52. A capture: tag assay according to claim 51 wherein the FcR is bound, and different epitopes of the said FcR are utilized for further binding and or antigenic functions.

53. A capture: tag assay according to claim 51 wherein the FcR is free.

54. An assay according to any one of claims 50 to 53 comprising the steps of washing away unbound material, adding biological fluid (e.g. serum), incubating for ½-16 hours, washing unbound materials, adding a second labelled anti-human Ig (labelled for example with radioisotope; enzyme particle or fluorochrome), incubating for 1-24 hours, washing and determining the quantity of labelled antibody bound.

55. A capture tag assay according to any one of claims 51 to 53 wherein said assay utilizes hybrid FcR as a standard or competitive inhibitor.

56. A method of regulating antibody production in vivo by binding a hybrid FcR according to any one of claims 2 to 30 to B cells.

57. The use of the method of claim 56 to control autoimmune disease.

58. The use of the method of claim 56 to control allergy reaction by affecting IgE control.

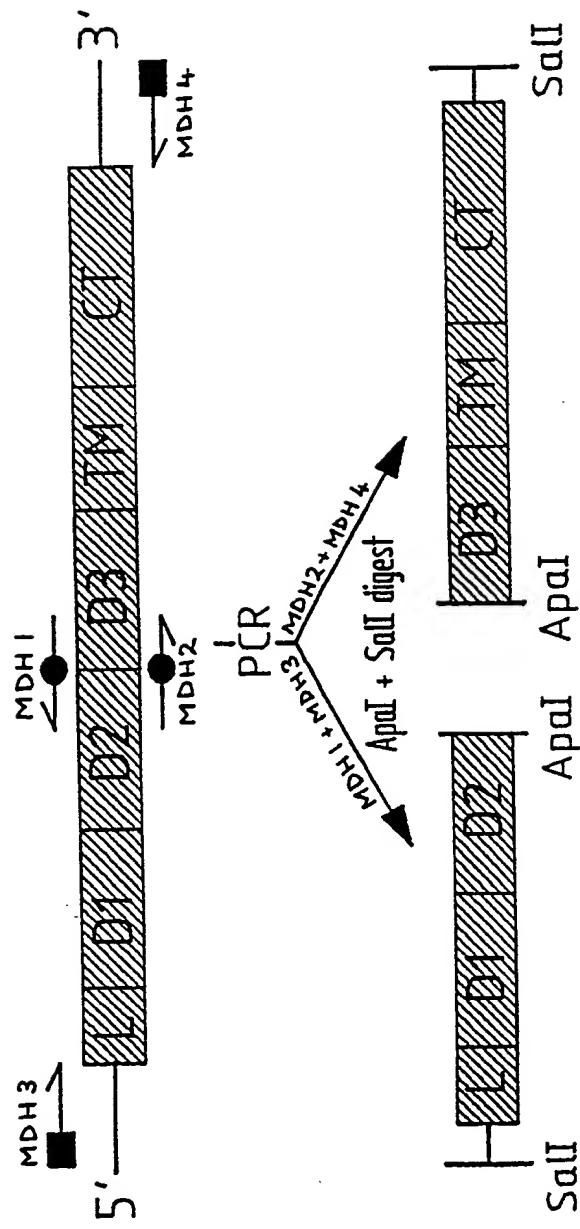
59. A method of regulating allergic reactions by reacting hybrid FcR according to any one of claims 2 to 30 with IgE in vivo.

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60. The use of the biological product of any one of claims 43 to 48 for plasmaphoresis to remove immune complexes or pathological antibodies.
61. The use of the biological product of any one of claims 43 to 48 in conjunction with biosensors.

FIGURE ONE

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FIGURE TWO

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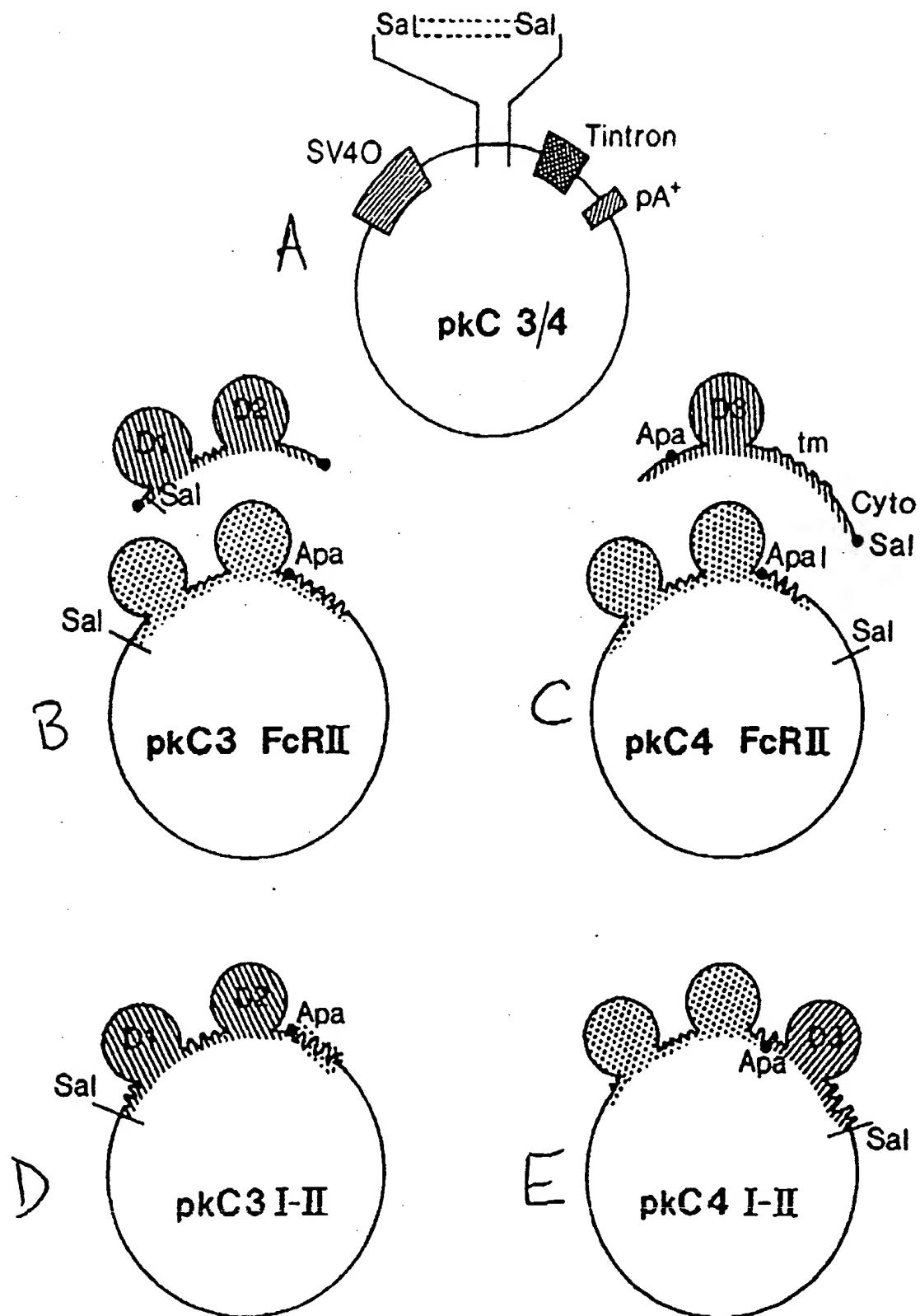


FIGURE THREE A

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Met Ile Leu Thr Ser Phe GLY Asp Met Trp Leu Leu Thr Thr Leu Leu Val Pro Val GLY GLY Glu Val Val
 TTT ~~GGGAC~~ ATG ATT CTT ACC AGC TTT GGA GAT GAC ATG TCG CTT CTA ACA ACT CCT GCA CTC TGG GTC GGT 70
 10 30 40 50 60 70 80
 Asn Ala Thr Lys Ala Val Ile Thr Leu Gln Pro Pro Val Ser Ile Phe Gln Lys Glu Asn Val Thr Leu Trp Cys Glu GLY Pro His
 AAT GCC ACC AAG GCT GCT GTC ACC TGG ATC ACC TGG CTC AGT ATT TTC CAG AAG GAA ATT GTC ACT TTA TGG TGT GAG GGG CCT CTC
 110 130 120 140 150 160 170 180
 Leu Pro GLY Asp Ser Thr Gln Trp Phe Ile Asn Gln The Ala Val Gln Ile Ser Thr Pro Ser Tyr Ser Ile Pro Glu Ala Ser Phe
 CTC CCT GGA GAC AGT TCC ACH CAA TGG TTT ATC AAC TGG 210 220 230 240 250 260 270
 190 200
 Gln Asp Ser GLY GLU Tyr Arg Cys Gln Ile GLY Ser Ser Met Pro Ser Asp Pro Val GLN Leu Gln Ile His Asn Asp Trp Leu Leu
 CTC GAC ACT GGC GAA TAC AGG TGT CAG ATA GAT TCC TCA ATG CCA AGT GAC CCT GCA CTC NAT GAT GAT GAT GTC CTA 350
 280 290 300 310 320 330 340 350
 Gln Ala Ser Arg Arg Val Leu Thr GLU GLY GLU Pro Leu Ala Leu Arg Cys His GLY TRP Lys Asn Val Val Val Val Phe
 CAG GGC TCC CGC AGA GTC CTC ACA GAA CCC CTG GCC 390 400 410 420 430 440 450
 370 380
 Tyr Arg Asn GLY Lys Ser Phe GLN Phe Ser Ser Asp Ser Gln Val Ala Ile Leu Val Val Tyr Asn Leu Ser His Ser GLY Ile Tyr His Cys
 TAT AGA ATT GGA NAA TCC TTT CAG TTT TCT TCA GAT TCG GAG GTC 460 470 480 490 500 510 520 530 540
 Ser GLY Thr GLY Arg His Arg Tyr Thr Ser Ala GLY Val Ser Ile Thr Val Val Lys GLY Pro Lys Ser Ser Arg Ser Leu Pro Val Leu Thr
 TCA GGC AGC GGA AGA CAC CGC TAC ACA TCT GCA GGA GTC ATC ACG GTC AAA GGG CCC AAG TCC AGC AGG TCT TTA CCA GTC ATT 610 620
 550 560 570 580 590 600 610 620
 Ile Val Ala Val Thr GLY Ile Ala Val Ala Ile Val Ile Ile Leu Val Ser Leu Val Tyr Leu Lys Lys Gln Val Pro Ala
 ATT GTC GCT GTC ACT GGG ATT GCT GCA GGC ATT GTR ATT AIC CTA GTC TCC TGG GTC TAT CTC AGG AAR ARG CAG GTT CCA GCT 720
 640 650 660 670 680 690 700 710 720
 Leu Pro GLY Asn Pro Asp His Arg GLU Met GLY GLU Thr Leu Pro GLU Val GLY GLU Ile Val GLN Pro Ser GLY GLY Ser Val Pro
 CTC CCA GGA AAC CCT GAT CAC AGG GAA ATT GGA AAC CCT CTT CCA GAG GAA GAT GTC ATT GCA GGT GAG TAC AGA CAG CCC TCT GGG GGC TCA GTC CCT
 730 750 760 770 780 790 800 810
 Val Ser Pro GLY Pro Ser GLY Leu GLU Pro Thr Ser Ser Pro Tyr Asn Pro Asp Leu GLN GLN Pro Lys Thr GLU Ala
 GTC AGC CCA GGG CCT CCA TCT GGA CTG GAG CCA ACA AGC AGC AGC 820 830 840 850 860 870 880 890 900
 Gln Asn Thr Ile Thr Tyr Ser Leu Leu Lys His Pro GLU Ala Leu Asp GLU Glu Thr GLU His Asp TYR GLN Asn His Ile ***
 GAG AAC ACG ATC ACC TAC TCA CTC CTC AAG CAT CCC GAA GTC TGG GAT GAA GCA GAG 910 920 930 940 950 960 970 980 990
 TGGCATTGGGAAAGCCAGGAAAGGCCAGGATCTAGTGTCTCCCTGCTGAGTAAAGGGATGCTCCAGCTTCCAGTCAAGCTCTGCTATGGATCCACACTGGCACTTAATTTACTGCGT
 1001 1011 1021 1031 1041 1051 1061 1071 1081 1091 1101 1111
 TACGGGATTGTTCCCATGGTGTACTAATGACTCCGATACCTACAGCTTCCAACTCAAGCTCTGCTATGGATCCACACTGGCACTTAATTTACTGCGT
 1121 1131 1141 1151 1161 1171 1181 1191 1201 1211 1221 1231
 TAGAGACTGCGAG
 1241

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FIGURE THREE B

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Met Glu Ser Asn TRP Thr Val His Val Phe Ser Arg Thr Leu Cys His Met
 CTCGAGACTCGTCCAGGGATCTGGATGGCTTCTACTGATCCCC ATG GAG AGC AAC TGG ACT GTC CAT GTC TGC TTC TCA CGG ACT TGG TGC TTG TGC CAT ATG 100
 50 30 40 50 60 70 80 90 100
 Leu Leu TEP Thr Ala Val Ile Asn Leu Ala Ala Gly Thr His Asp Leu Pro Lys Leu Val Val Lys Leu Glu Pro Pro TRP Ile Gln Val
 CTA CTC TGG AGC GCC GTC CAA ATT CTT GCT GCG ACT CAT GAT GCT 113 123 133 143 153 163 173 183 193
 Leu Lys Glu Asp Thr Val Thr Leu Thr Cys Glu Gly Thr His Asn Pro Gly Asn Ser Ser Thr Gln Trp Phe His Asn GLY Arg Ser Ile
 CTC AAG GAA GAC AGC GTG ACA CTC GCA TGC AAC GGG ACC CCT GAG AAC TAC TCT TCA TCC CAC ATT GGG AGG TCC ATC 203
 213 223 233 243 253 263 273 283
 Arg Ser Gln Val Ala Ser Tyr Thr Phe Lys Ala Thr Val Cys Gln Met Glu Gln Thr Arg Leu Ser
 CGG AGC CAG GTC CAA ATT GGC AGC TAC ACG 313 323 333 343 353 363 373
 Asp Pro Val Asp Leu Gly Val Ile Ser Asp TRP Leu Leu Gln Thr Pro Gln Leu Val Phe Leu Glu Gly Glu Thr Leu Arg
 GAC CCT GTC GAT CTC GGA GTG ATT TCT GAC TGG CTC 393 403 413 423 433 443 453 463
 Cys His Ser TRP Arg Asn Lys Leu Leu Arg Ile Ser Phe Phe His Asn Gln Lys Ser Val Arg Tyr His Tyr Ser Ser Asn Phe
 TGC CAT AGC TGG AGG AAC AAA CTA CTG AAC AGG ATC TCG TTC TIC CAT GAA AAA 473 493 503 513 523 533 543 553
 Ser Ile Pro Lys Ala Asn His Ser His Ser Gln Asp Tyr Tyr Cys Lys Gln Ser Leu Gly Arg Thr Leu His Gln Ser Lys Pro Val Thr
 TCT ATC CCC AAA GCC AAC CAC AGT CAC AGT GAC TAC TAC TGC AAA GCA AGT CTA GGA AGG ACA CTC CAG TCC AAG CCT GTC ACC 563
 573 583 593 603 613 623 633 643
 Ile Thr Val Gln Gly Pro Phe Thr Thr Pro Val Leu Arg Ala Ser Val Ser Pro Phe Pro Gln Gly Ser Leu Val Thr Leu Asn Cys
 ATC ACT GTC CAA GGG CCC TTC ACC ACG GCA TCC GTG CTC AGA 673 683 693 703 713 723 733
 Glu Thr Asn Leu Leu Gln Arg Pro Gly Leu Gln Leu His Phe Ser Tyr Val Lys Ile Leu Glu Tyr Arg Asn Thr Ser
 GAG AGC AGT TTG CTC CTC CAG AGA CCC GGC TTA CAG 743 753 763 773 783 793 803 813 823
 Ser Glu Tyr His Ile Ala Arg Ala Glu Arg Glu Asp Ala GLY Phe Tyr Tyr Cys Gln Val Ala Thr Glu Asp Ser Ser Val Leu Lys Arg
 TCA GAG TNC CAT ATA GCA AGG GCG GAA AGC GAA GAT GCT GGA TTC TAC TCC TAC CAC TCC TAC TAC AAC TAA GCA TCT GAG AAC ACA 833
 843 853 863 873 883 893 903 913
 Ser Pro Glu Leu Gln Val Leu Gly Pro Gln Ser Ser Ala Pro Val Trp Phe His Ile Leu Phe Tyr Leu Ser Val Gly Ile Met
 AGC CCT GAG TTC GAG CTC CAA GTC 923 933 943 953 963 973 983 993 1003
 Phe Ser Leu Asn Thr Val Leu Tyr Val Lys Ile His Arg Leu Gln Arg Glu Lys Tyr Asn Leu Gln Val Pro Leu Val Ser Glu Gln
 TTT TCG TTG AAC AGC GTC CTC TAT GTC AAA ATA AAC CTC AGC CAG AAC TAC AAC TAA GCA TCA TCC TAC AAC TAA GCA TCT GAG CAG 1013
 1023 1033 1043 1053 1063 1073 1083 1093
 Gly Lys Lys Ala Asn Ser Phe Gln Gln Val Arg Ser Asp GLY Val Tyr Glu Glu Val Thr Ala Ser Gln Thr Pro Lys Glu
 GGA AAG AAA GCA ATT TCC TTT CAG CAA ATT AGC GAT GGC TAT GAA GAA GCA TCA ACA GCC ACT GCG AGC CAG AAC ACA CCA AAA GAA 1103
 1113 1123 1133 1143 1153 1163 1173 1183
 Ala Pro Asp GLY Pro Arg Ser Ser Val Gln Asp CYS Gly Pro Glu Gln Pro Glu Gln Pro Glu Pro Leu Pro Pro Ser Asp Ser
 GCG CCC GAT GGA CCT CGA AGC TCA GTG GGT GAC TGT GCA CCC GAG CAG CCT GAA CCC CTT CCT CCC AGT GAC AGT ACT GGG GCA CAA ACT 1193
 1203 1213 1223 1233 1243 1253 1263 1273
 Ser Gln Ser ***
 TCC CAA AGT TGA CCCTGAAACTGTGGACCATGGCATGAACTCTTAATAAGCAATAATAGCTAGGGAAAGCTCTAGGGATCCCCGGGTTGGGGAA 12

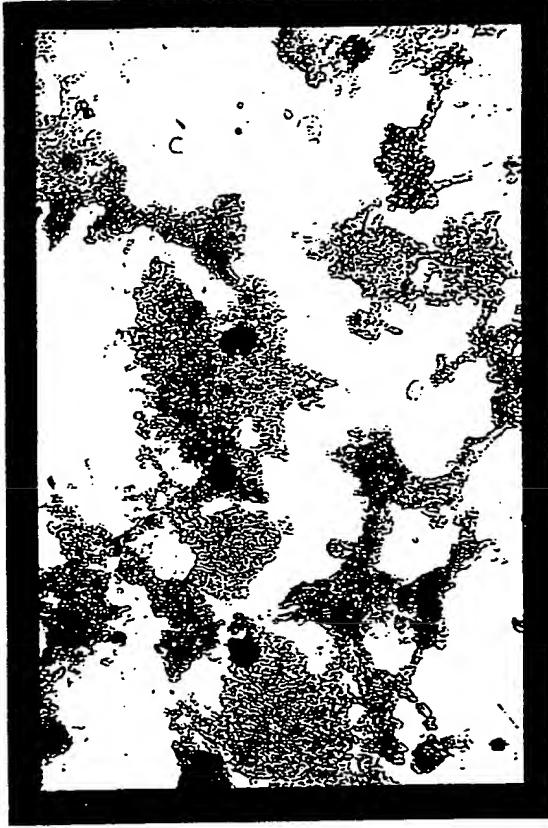
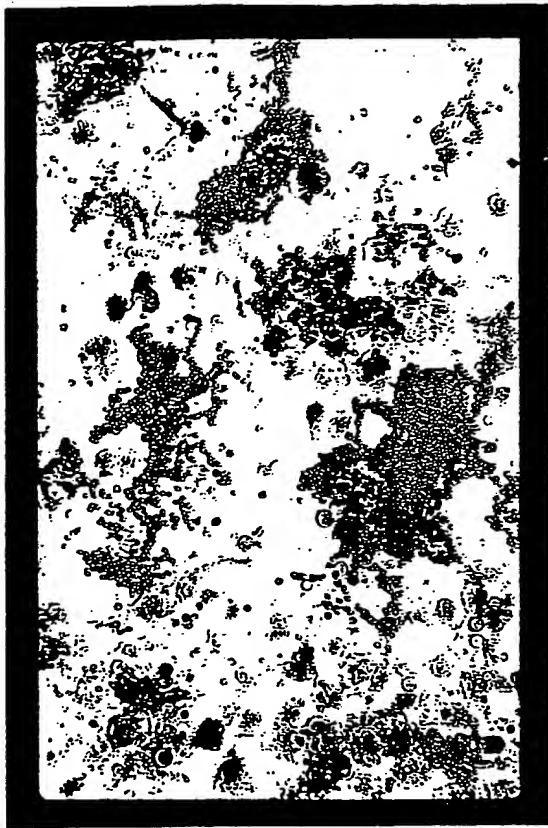
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FIGURE FOUR (A-D)

4a

4c

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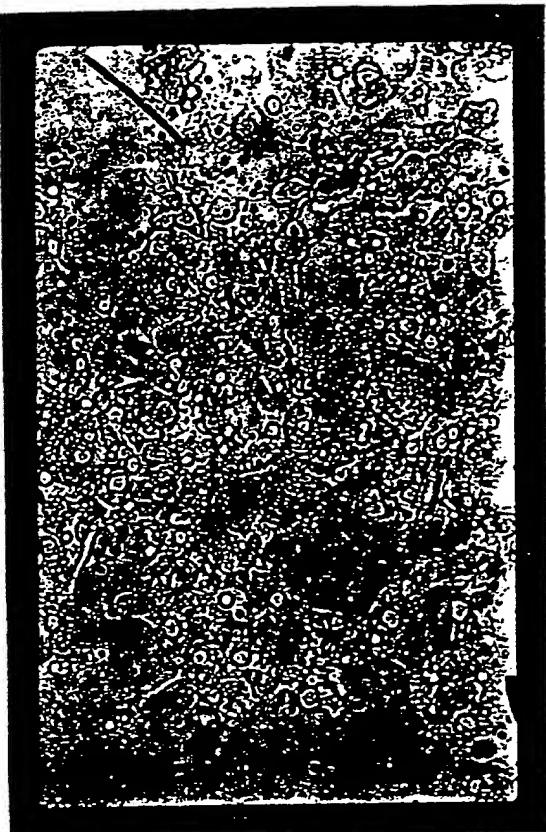
4b

4d



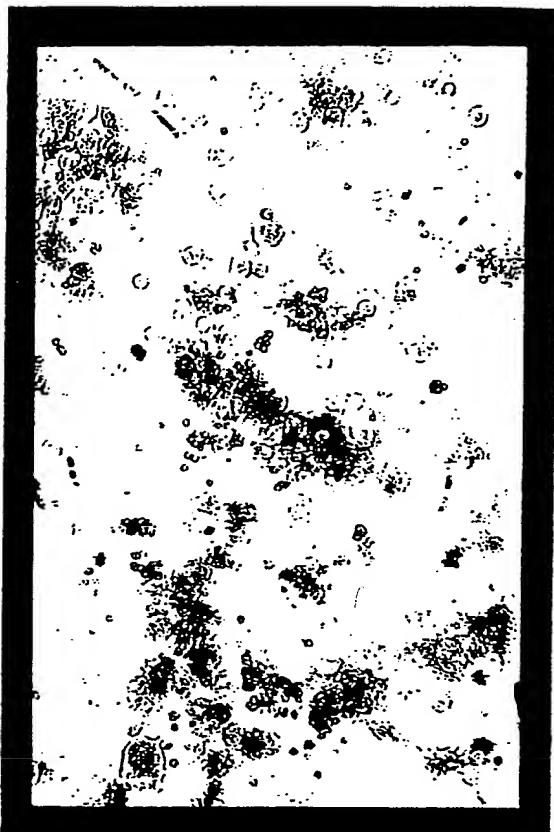
FIGURE FOUR (E-H)

4e



4g

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4f



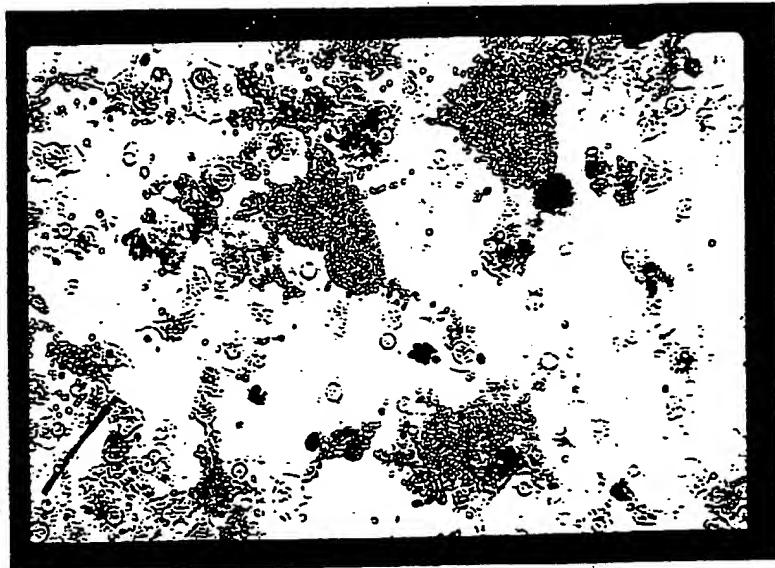
4g



FIGURE FOUR (I-J)

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4i



4j

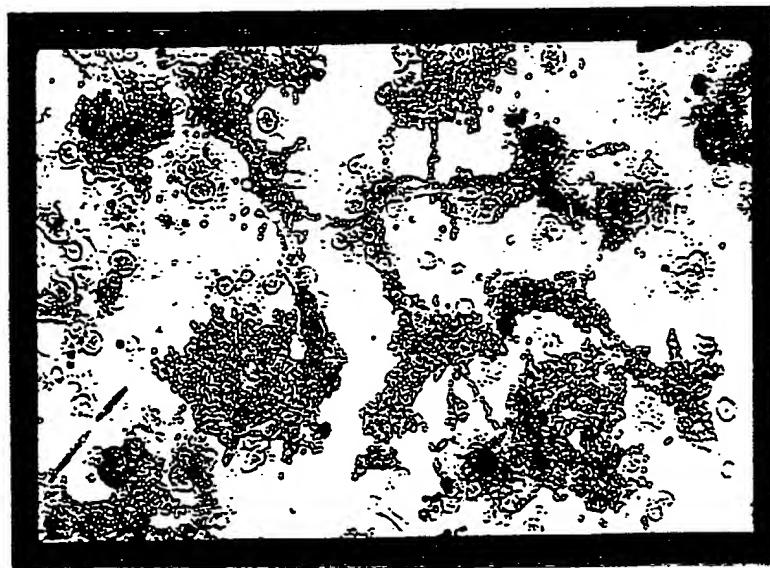


FIGURE FIVE

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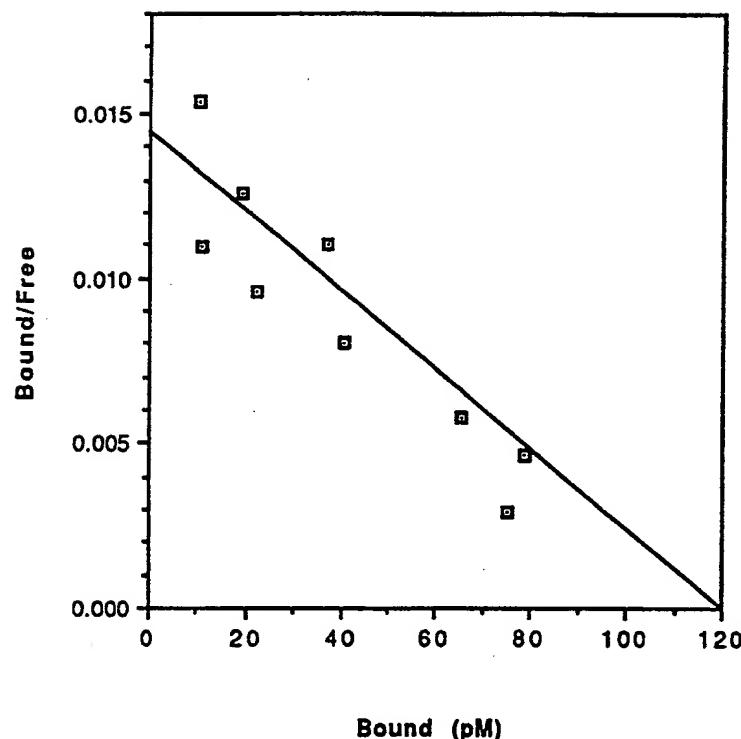


FIGURE SIX

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FIGURE SEVEN

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FIGURE EIGHT (A-D)

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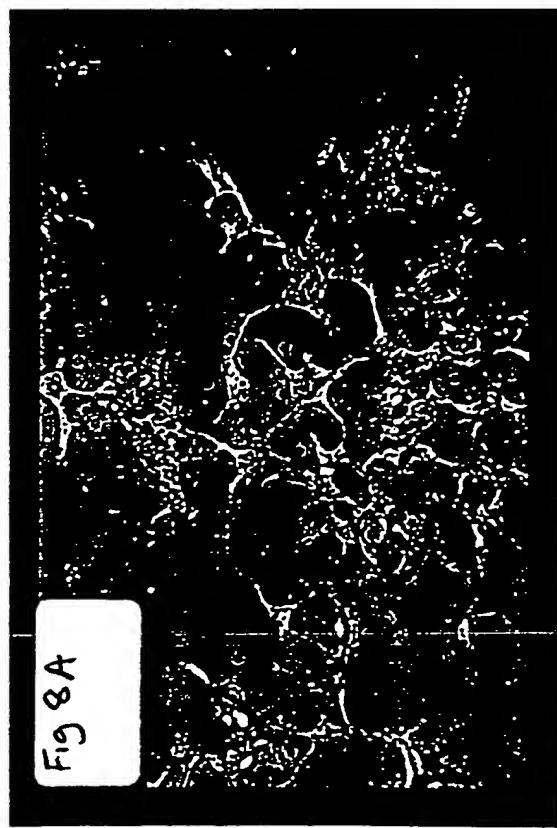
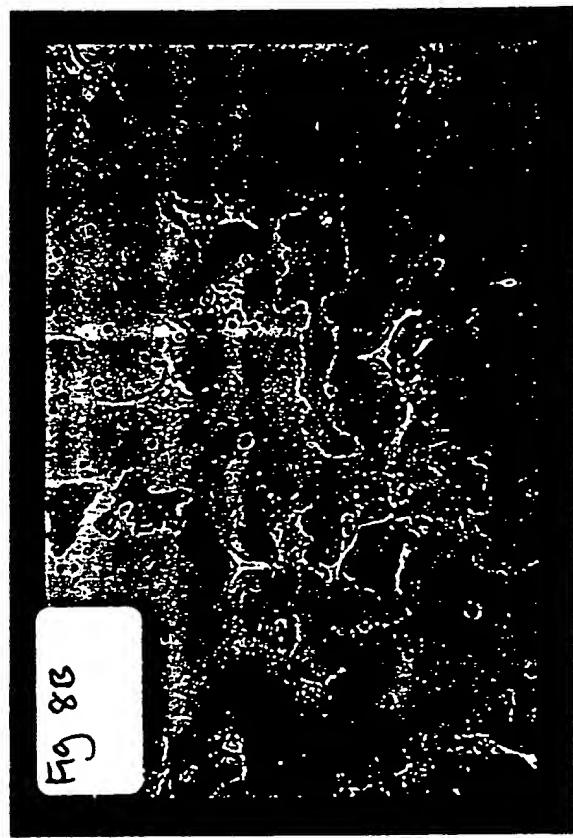
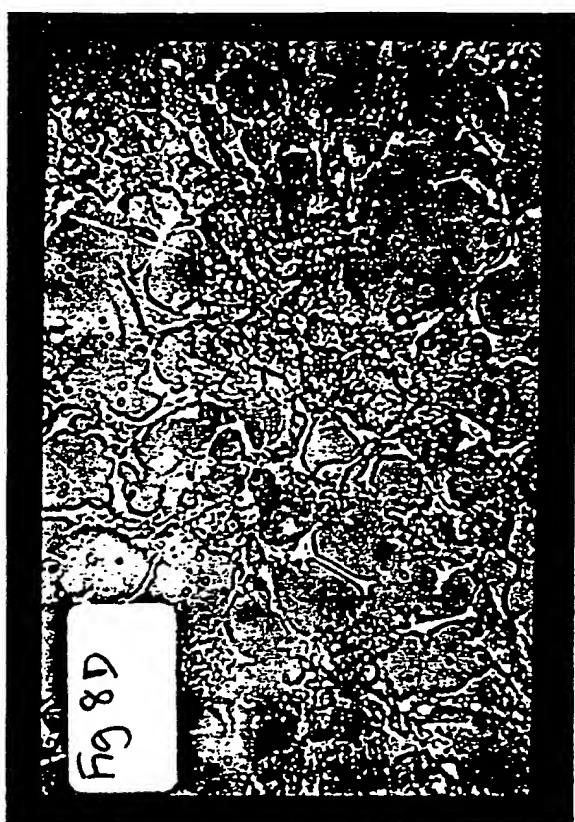


FIGURE EIGHT (E-H)

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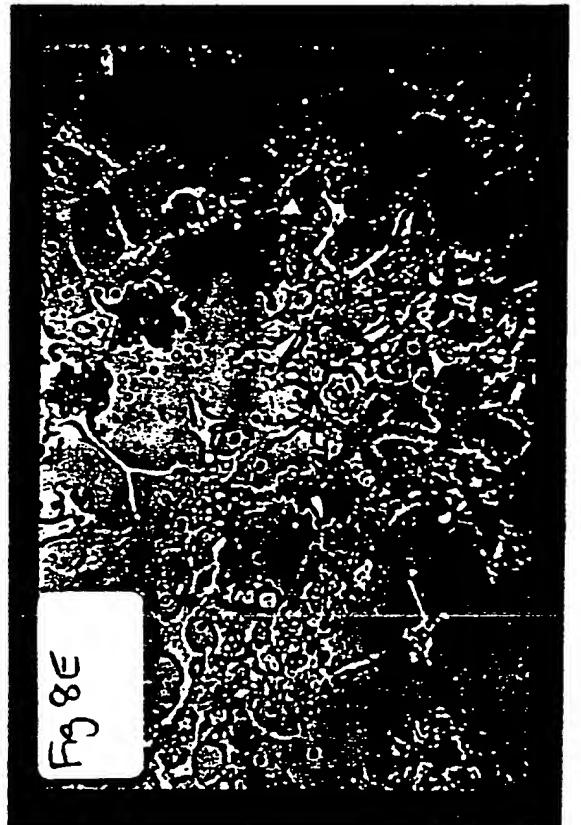
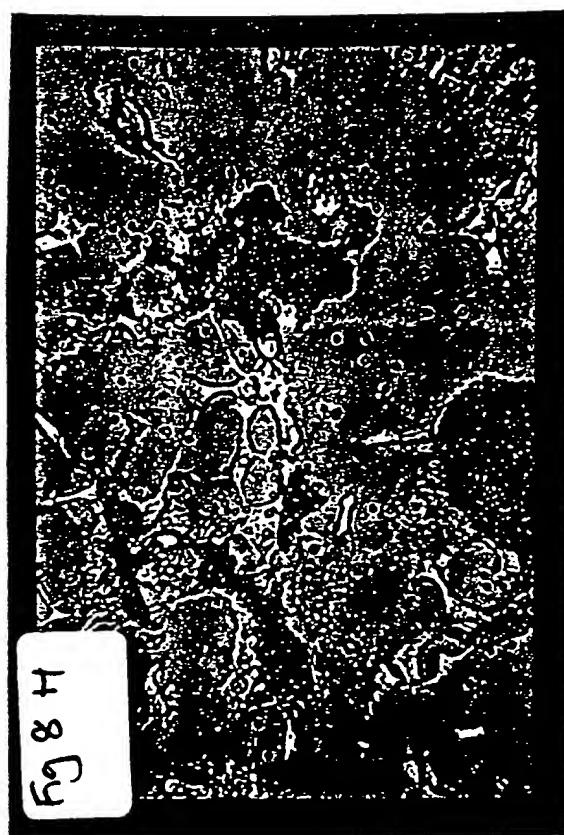
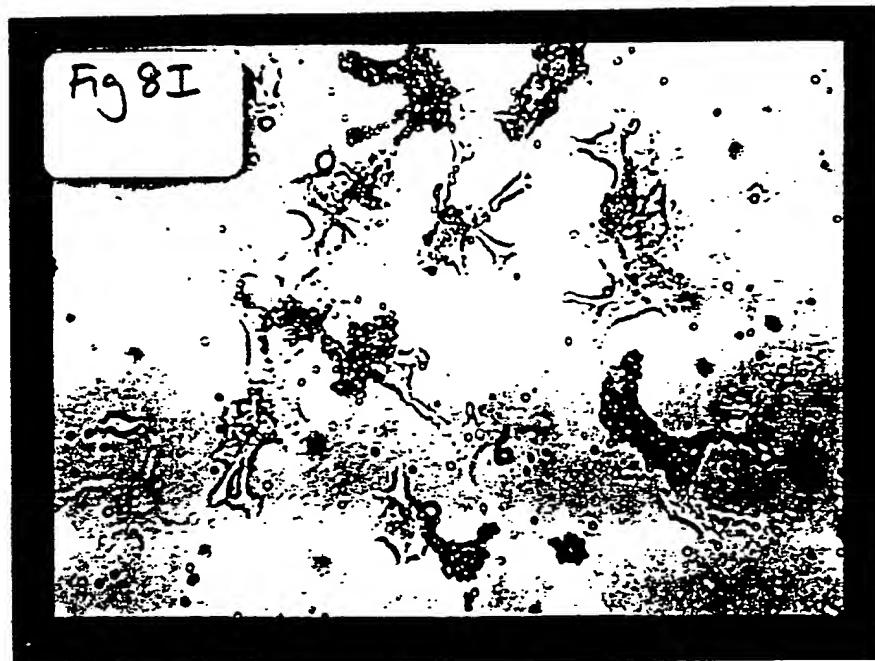


FIGURE EIGHT (I-J)

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FIGURE NINE

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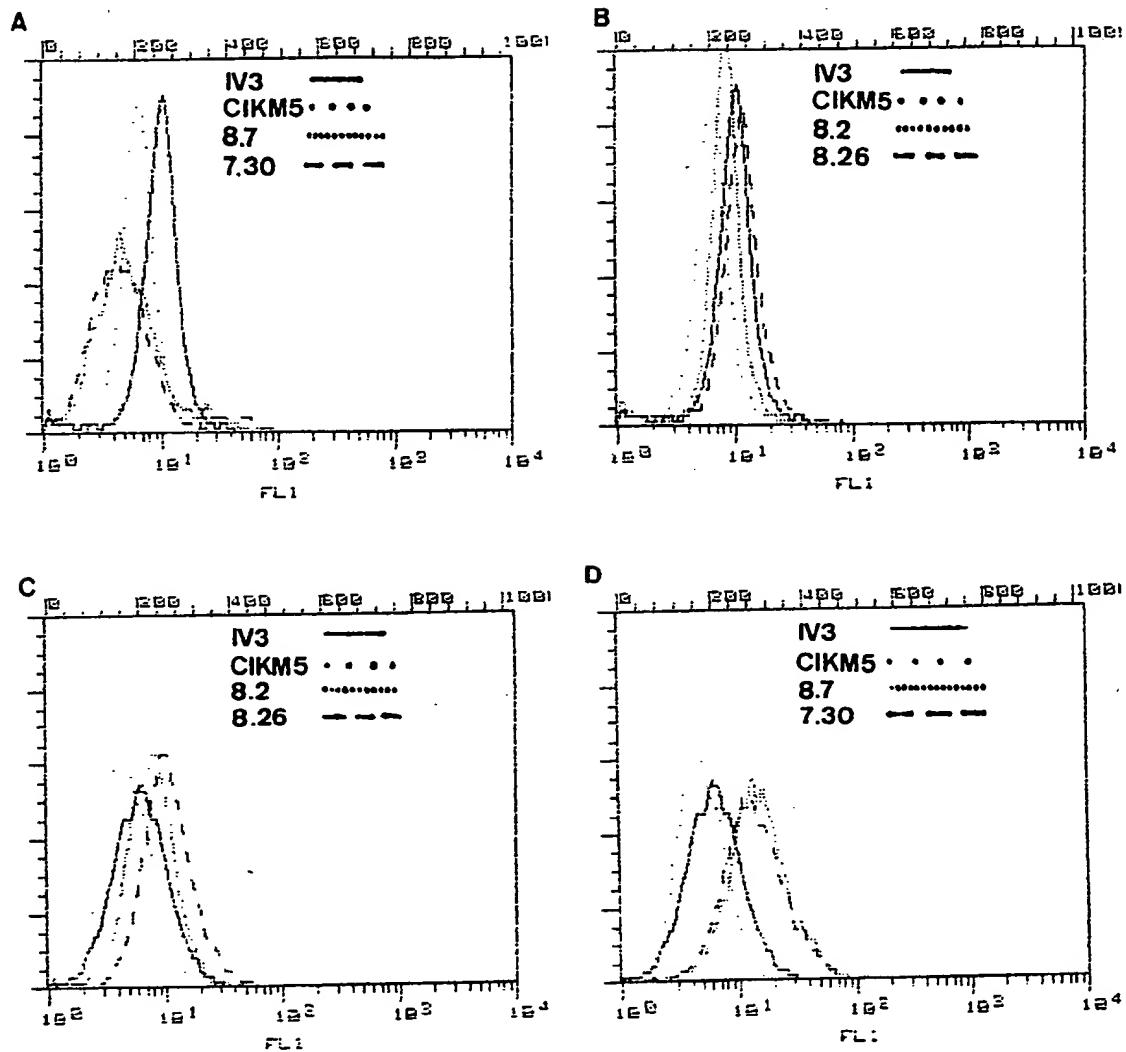


FIGURE TEN

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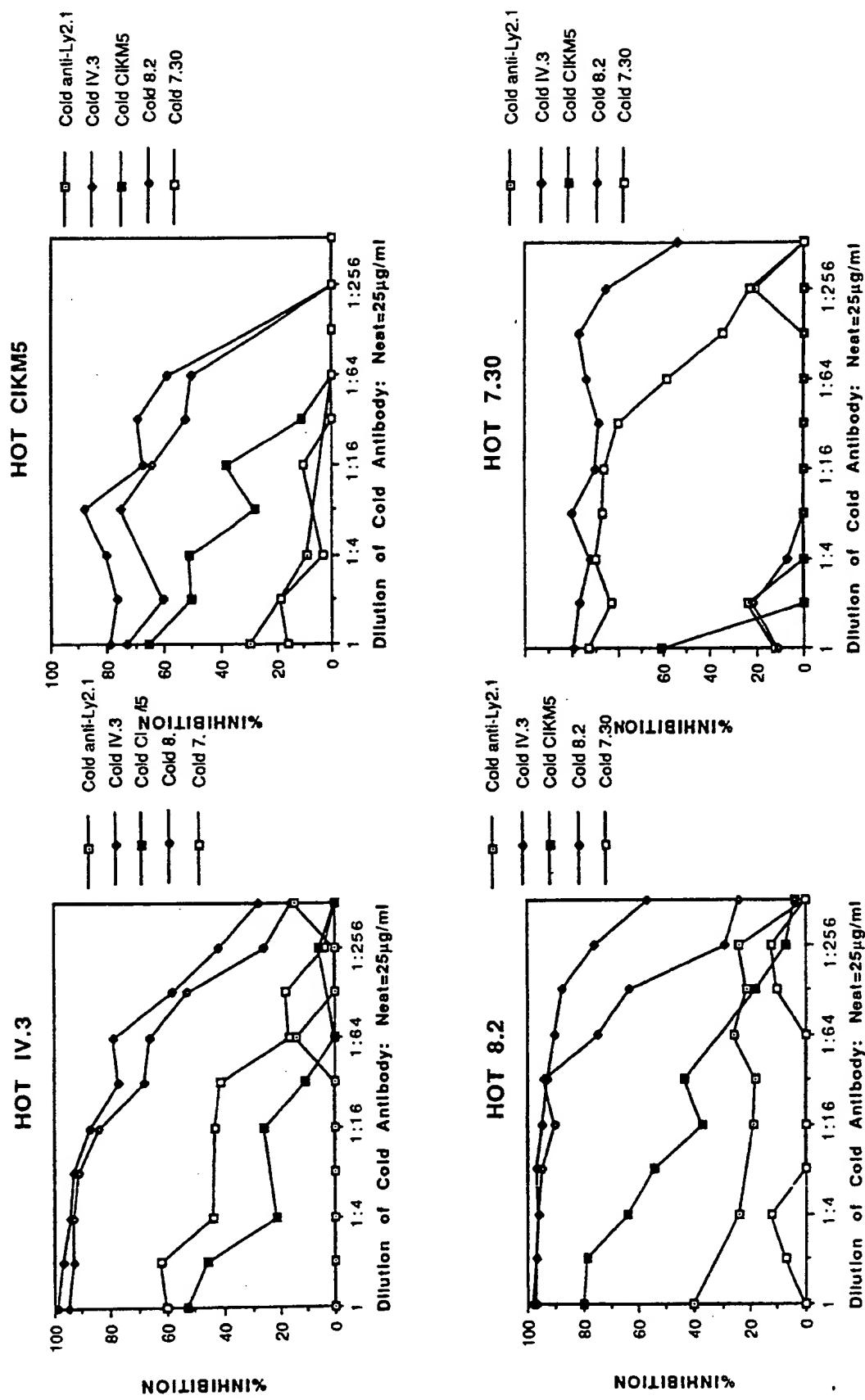


FIGURE ELEVEN A

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17.10.90 Immunpptn NP-40 O/N Exposure
10% reducing

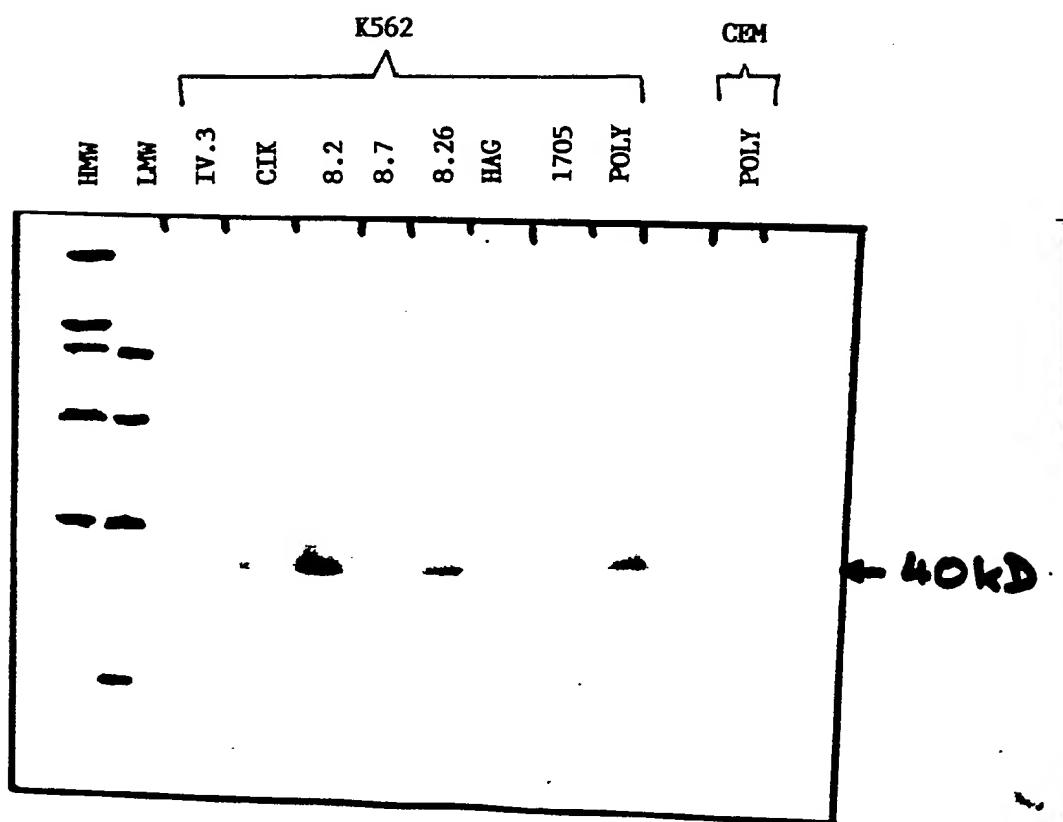
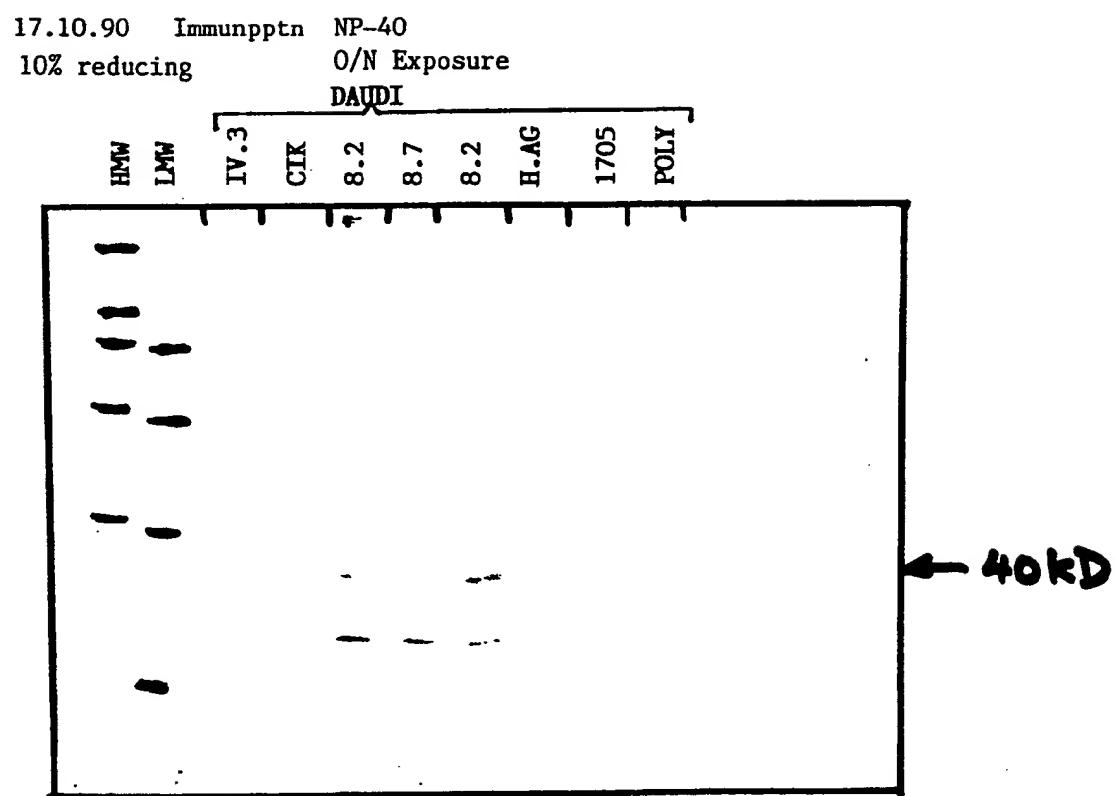
**SUBSTITUTE SHEET**

FIGURE ELEVEN B

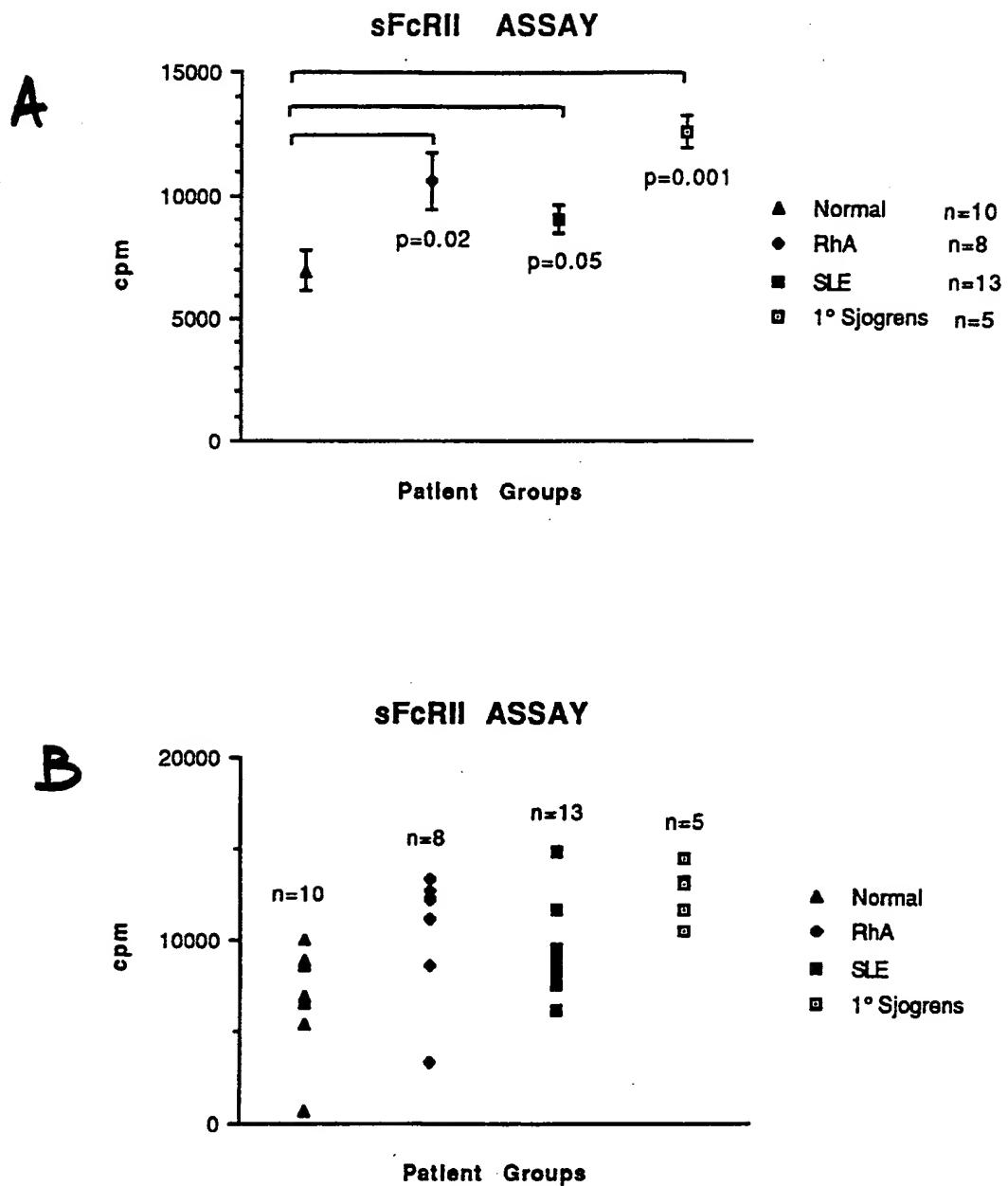
17/18



SUBSTITUTE SHEET

FIGURE TWELVE

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00513

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.⁵ C07K 15/06, 15/08, 15/12, C12N 15/13, C12P 21/08, G01N 33/563

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPAT : Keywords : "Immunoglobulin", "Receptor", "Ig", "Fc", "FcR" USPA : Keywords : As above

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

AU: C07K 15/06, 15/08, 15/12, 13/00, C07G 7/00, C12N 15/12, 15/13, C12P 21/08
Chemical Abstracts : Keywords : "Immunoglobulin" or "Ig" and "Binding Molecule";
"Fc Receptor" or "FcR" and "Molecule"

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
A	AU,A, 32842/89 (SCHERING BIOTECH CORPORATION) 5 July 1989 (05.07.89)	
A	AU,A, 27830/88 (KISHIMOTO, T.) 13 July 1989 (13.07.89)	
A	AU,A, 82701/87 (MEMORIAL SLOAN-KETTERING CANCER INSTITUTE) 23 June 1988 (23.06.88)	
A	AU,A, 74881/87 (THE UNIVERSITY OF MELBOURNE) 4 February 1988 (04.02.88)	
A	AU,A, 63858/86 (MEDICAL BIOLOGY INSTITUTE) 14 April 1988 (14.04.88)	
A	EP,A, 269455 (TAKEDA CHEMICAL INDUSTRIES, LTD) 1 June 1988 (01.06.88)	

(continued)

* Special categories of cited documents: 10	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"		document defining the general state of the art which is not considered to be of particular relevance
"E"		earlier document but published on or after the international filing date
"L"		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"		document referring to an oral disclosure, use, exhibition or other means
"P"		document published prior to the international filing date but later than the priority date claimed
	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
	"Z"	document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 4 February 1991 (04.02.91)	Date of Mailing of this International Search Report 11 February 1991
International Searching Authority Australian Patent Office	Signature of Authorized Officer K. AYERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Hibbs, M.L. et al. Proc. Natl. Acad. Sci. USA, Volume 85, issued April 1988, "Molecular cloning of a human immunoglobulin G Fc receptor" see pages 2240-2244	
A	Shimizu, A. et al. Proc. Natl. Acad. Sci. USA, Volume 85, issued March 1988, "Human and rat mast cell high-affinity immunoglobulin E receptors: Characterization of putative α -chain gene products" see pages 1907-1911	
P,A	Allen, J.M. and B. Seed. Science, Volume 243, issued 20 January 1989, "Isolation and expression of functional high-affinity Fc receptor complementary DNAs" see pages 378-381	
A	Hibbs, M.L. et al. Proc. Natl. Acad. Sci. USA, Volume 83, issued September 1986, "The murine Fc receptor for immunoglobulin: Purification, partial amino acid sequence, and isolation of cDNA clones" see pages 6980-6984	

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSearchABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

| Remark on Protest

| [] The additional search fees were accompanied by applicant's protest.

| [] No protest accompanied the payment of additional search fees.

ANXEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00513

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members				
AU 32842/89	EP 319307	WO 8905351			
AU 27380/88	DK 7124/88 HU 50498 NO 885687 ZA 8809506	EP 321842 IL 88743 PT 89299	FI 885873 JP 2000443 EP 321601		
AU 82701/87	WO 8803172				
AU 74881/87	EP 268636	JP 63503386	WO 8707277		
EP 269455	JP 63246398				